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(54) Title: COMPOSITIONS FOR INDUCING AN IMMUNE RESPONSE

(57) Abstract: Priming and boosting compositions and methods for inducing an immune response, e.g. a CD8+ T cell response, to an antigen e.g. a tumour antigen, for example a melanoma antigen. Methods of treating metastasis of melanoma in mammals. DNA plasmids and/or poxvirus, e.g. modified vaccinia virus Ankara, as priming and/or boosting compositions for inducing immune responses to antigen epitopes.

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Compositions for Inducing an Immune Response

This invention relates to the inducing and/or boosting of an immune response (e.g. a CD8+ T cell immune response) against an antigen, normally a tumour antigen e.g. a melanoma tumour antigen, in an individual e.g. a mammal.

There is a clear need for the development of improved methods of inducing immune responses to diseases in an individual.

The present invention is directed to a method of inducing an immune response against an (one or more) antigen (e.g., a tumour antigen) in a mammal (e.g., human), which comprises administering to the mammal a priming composition (e.g., a DNA plasmid) comprising a source of one or more epitopes of the target antigen; a first boosting composition comprising a source of one or more epitopes of the target antigen (e.g., a non-replication or replication-impaired poxvirus such as MVA), including at least one epitope which is the same as an epitope of the priming composition; and a second boosting composition comprising a source of one or more epitopes of the target antigen, including at least one epitope which is the same as an epitope of the priming composition, at a dose that is higher than the dose of the first boosting composition.

In one aspect, the invention is a composition comprising a priming composition, a first boosting composition and a second boosting composition for sequential administration to an individual to induce an immune response against an antigen; wherein

the priming composition comprises a source of one or more epitopes of the antigen;

the first boosting composition comprises a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming composition; and

the second boosting composition comprises a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming

composition, wherein the source of epitopes of the first boosting composition is different from the source of epitopes of the second boosting composition; and wherein

the second boosting composition is for administration at a dose that is higher than the dose of the first boosting composition.

Another aspect of the invention is use of a priming composition, a first boosting composition and a second boosting composition in the manufacture of a medicament for sequential administration to an individual to induce an immune response against an antigen; wherein

the priming composition comprises a source of one or more epitopes of the antigen;

the first boosting composition comprises a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming composition; and

the second boosting composition comprises a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming composition, wherein the source of epitopes of the first boosting composition is different from the source of epitopes of the second boosting composition; and wherein

the second boosting composition is for administration at a dose that is higher than the dose of the first boosting composition.

Another aspect of the invention is use of the first boosting composition and the second boosting composition in the manufacture of a medicament for sequential administration to boost a pre-existing immune response to an antigen in an individual to whom the priming composition was previously administered, wherein the second boosting composition is for administration at a dose that is higher than the dose of the first boosting composition.

In one embodiment, the priming composition is administered as, or comprises, a viral vector which is derived from a virus other than a non-replicating of replication-impaired poxvirus.

In another embodiment, the source of the epitopes of the first boosting composition and the second boosting composition are different and the second boosting

composition is administered at, or is for administration at, a higher than the dose of the first boosting composition.

In a particular embodiment, the source of the epitopes of the first boosting composition and the second boosting composition are the same except that the second boosting composition is administered at a dose that is higher than the dose of the first boosting composition. Accordingly, a further aspect of the invention is use of a priming composition and a boosting composition in the manufacture of a medicament for sequential administration to an individual to induce an immune response against an antigen; wherein

the priming composition comprises a source of one or more epitopes of the antigen; and

the boosting composition comprises a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming composition; and wherein

the boosting composition is for sequential administration as a first boosting composition and a second boosting composition, wherein the second boosting composition is for administration at a dose that is higher than the dose of the first boosting composition.

Another aspect of the invention is use of the boosting composition in the manufacture of a medicament for boosting a pre-existing immune response to an antigen in an individual to whom the priming composition was previously administered, wherein the boosting composition is for sequential administration as a first boosting composition and a second boosting composition, wherein the second boosting composition is for administration at a dose that is higher than the dose of the first boosting composition.

In yet another embodiment, the second boosting composition is at a dose that is about four times greater than the dose of the first boosting composition.

In a further aspect, the invention relates to a kit for inducing an immune response against an antigen in an individual, wherein the kit comprises:

a) a priming composition comprising a source of one or more epitopes of the target antigen;

b) a first boosting composition comprising a source of one or more epitopes of the target antigen, including at least one epitope which is the same as an epitope of the priming composition; and

c) a second boosting composition comprising a source of one or more epitopes of the target antigen, including at least one epitope which is the same as an epitope of the priming composition; and

instructions for administering the second boosting composition of c) at a dose that is higher than the dose of the boosting composition of b).

In another aspect, the invention relates to a kit for boosting a pre-existing immune response to an antigen in an individual to whom a priming composition comprising a source of one or more epitopes of the antigen was previously administered, wherein the kit comprises:

a) a first boosting composition comprising a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming composition; and

b) a second boosting composition comprising a a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming composition; and

instructions for administering the first composition, and then administering the second composition of at a dose that is higher than the dose of the first composition, for example at a dose that is about four times greater than the dose of the first boosting composition.

A kit according to the invention can comprise two doses of each composition.

In preferred embodiments of the invention, the priming composition is or comprises a DNA plasmid. Preferably, the boosting composition (e.g. first and/or second boosting composition) is or comprises a non-replicating or replication-impaired poxvirus, preferably a Modified Vaccinia Virus Ankara (MVA).

The source of epitopes in the priming composition is preferably different from the source of epitopes in the boosting composition or boosting compositions. For example, where a non-replicating or replication-impaired poxvirus is used in the boosting

composition or boosting compositions, the priming composition can be or can comprise a viral vector derived from a virus other than a non-replicating or replication-impaired poxvirus. Heterologous prime-boost is discussed further below.

In a further aspect, the invention relates to use of a recombinant replication-deficient or replication-impaired poxvirus, e.g. MVA, comprising a heterologous polynucleotide encoding one or more epitopes of an antigen in the manufacture of a medicament for inducing an immune response against the antigen in an individual, wherein the medicament is for sequential administration at a first dose and at a second dose, and wherein the second dose is higher than the first dose. In one embodiment, the immune response of the individual has not been primed with a priming composition, such as a DNA plasmid, encoding one or more epitopes of the antigen, prior to administration of the recombinant replication-deficient or replication-impaired poxvirus.

The invention also relates to use of a first composition and a second composition in the manufacture of a medicament for sequential administration to an individual to induce an immune response against an antigen, wherein

the first composition comprises a recombinant replication-deficient or replication impaired poxvirus, e.g. MVA, comprising a heterologous polynucleotide encoding one or more epitopes of the antigen; and

the second composition comprises a recombinant replication-deficient or replication impaired poxvirus, e.g. MVA, comprising a heterologous polynucleotide encoding one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the first composition; and wherein

the second composition is for administration at a dose than is higher than the dose of the first composition.

In one embodiment, the immune response of the individual has not been primed with a priming composition, such as a DNA plasmid, encoding one or more epitopes of the antigen, prior to administration of the first and second composition.

In another aspect, the invention is a kit for inducing an immune response against an antigen in an individual, wherein the kit comprises:

a) a first composition comprising a recombinant replication-deficient or replication impaired poxvirus, e.g. MVA, comprising a heterologous polynucleotide encoding one or more epitopes of the antigen; and

b) a second composition comprising a recombinant replication-deficient or replication impaired poxvirus, e.g. MVA, comprising a heterologous polynucleotide encoding one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the first composition; and

instructions for administering a dose of the first composition and then administering a dose of the second composition, wherein the dose of the second composition is higher than the dose of the first composition.

In one embodiment, the immune response of the individual has not been primed with a priming composition, such as a DNA plasmid, encoding one or more epitopes of the antigen, prior to administration of the first and second composition.

The kit may comprise two doses of each of the first composition and the second composition. In one embodiment, the instructions are for administering the second composition at a dose that is about four times greater than the dose of the first composition.

The first and the second composition may be the same or different. Unless otherwise indicated by context, features and embodiments described herein for the first boosting composition may apply to the first composition, and features and embodiments described herein for the second boosting composition may apply to the second composition.

Suitable administration regimens, including dosage, mode and sequence of administration, are described elsewhere herein. The first composition may be administered as described herein for the first boosting composition, and the second composition may be administered as described herein for the second boosting composition.

The present invention is also directed to a method of treating melanoma in a mammal, which comprises administering to the mammal a priming composition

comprising a source of one or more epitopes of melanoma; a boosting composition (a first boosting composition) comprising a source of one or more epitopes of melanoma, including at least one epitope which is the same as an epitope of the priming composition, wherein the source of melanoma epitopes is a non-replicating or replication impaired recombinant poxvirus; and the boosting composition (a second boosting composition) at a dose that is higher than the dose of the first boosting composition, with the proviso that if the source of epitopes in the priming composition is a viral vector, the viral vector in the boosting composition is derived from a different virus.

In another aspect, the invention is use of a priming composition and a boosting composition in the manufacture of a medicament for sequential administration to a mammal to treat melanoma; wherein

the priming composition comprises a source of one or more epitopes of melanoma; and

the boosting composition comprises a source of one or more epitopes of melanoma, including at least one epitope which is the same as an epitope of the priming composition, wherein the source of melanoma epitopes is a non-replicating or replication impaired recombinant poxvirus; and wherein

the boosting composition is for sequential administration as a first boosting composition and a second boosting composition, wherein the second boosting composition is for administration at a dose that is higher than the dose of the first boosting composition;

with the proviso that if the source of epitopes in the priming composition is a viral vector, the viral vector in the boosting composition is derived from a different virus.

The medicament may be administered in accordance with an administration regime described elsewhere herein.

In another aspect, the invention is a kit for treating melanoma in a mammal, wherein the kit comprises

a) a priming composition comprising a source of one or more epitopes of melanoma;

b) a boosting composition comprising a source of one or more epitopes of melanoma, including at least one epitope which is the same as an epitope of the priming composition,

wherein the source of melanoma epitopes is a non-replicating or replication impaired recombinant poxvirus; and

instructions for administering the boosting composition of b) at a specified dose, followed by administering the boosting composition of b) again at a higher dose than the specified dose;

with the proviso that if the source of epitopes in a) is a viral vector, the viral vector in b) is derived from a different virus.

The instructions may be for administering the compositions in accordance with a regimen described elsewhere herein.

In one embodiment, the priming composition is a DNA plasmid (e.g., pSG.Mel3). In another embodiment, the non-replicating or replication-impaired recombinant poxvirus is a Modified Vaccinia Virus Ankara (MVA) (e.g., MVA.Mel3). In a particular embodiment, the immune response induced is a CD8+ T cell immune response. In another embodiment, the melanoma is a stage III or stage IV melanoma. In yet another embodiment, tumour regression occurs after the second boosting composition is administered.

The present invention is also directed to isolated plasmid comprising the polyepitope string of SEQ ID NO: 2. In addition, the invention is directed to an isolated recombinant replication-deficient poxvirus (e.g., MVA) comprising the polyepitope string of SEQ ID NO: 2.

To the inventors knowledge, this is the first disclosure that prime-boost regimens with high doses of replication deficient poxvirus boost can induce immune responses in a dose-responsive manner and that high rates of immune responses, linked to survival, are observed with two boosts of 5×10^8 pfu or higher.

This invention therefore further provides a method of inducing an immune response against melanoma in a mammal, which comprises the steps of:

a) administering to the mammal a priming composition comprising at least 4 mg of a DNA plasmid expressing a melanoma epitope

b) administering a boosting composition to the mammal comprising at least 1 x 10^9 pfu of a replication-deficient poxvirus expressing the same melanoma epitope.

In specific embodiments, the priming composition and/or the boosting composition are administered in two or more separate doses, for example 2 doses of at least 2 mg of the DNA plasmid, followed by 2 doses of at least 5 x 10^8 pfu of the replication-deficient poxvirus.

Without limitation the DNA plasmid could also be administered in 1 dose of 4 mg, 2 or 3 doses of 4 mg, 3 or 4 doses of 2 mg, 2, 3 or 4 doses of 3 mg, or other combinations.

Without limitation the replication-deficient poxvirus could also be administered in 1 dose of 1 x 10^9 pfu, 2 or 3 or more doses of 1 x 10^9 pfu, one or more doses of 2 x 10^9, or up to 10^{10} pfu, 3 or 4 doses of 5 x 10^8 pfu, or other combinations.

In one embodiment, the melanoma epitope is a Melan-A melanoma epitope.

In a preferred embodiment, the melanoma epitope is a CD8+ T cell epitope and the immune response is a CD8+ T cell immune response.

In a more preferred embodiment, the melanoma epitope comprises the amino acid sequence ELAGIGILTV.

In a more preferred embodiment, the DNA plasmid expresses the amino acid sequence of SEQ ID No. 2

In a particularly preferred embodiment, the DNA plasmid expresses the amino acid sequence of SEQ ID No. 2 under the CMV promoter.

In a preferred embodiment, the replication-deficient poxvirus is MVA

In the most preferred embodiment, the replication-deficient poxvirus expresses the amino acid sequence of SEQ ID No. 2

In a particularly preferred embodiment, the replication-deficient poxvirus expresses the amino acid sequence of SEQ ID No. 2 from an insertion site in the TK locus under the control of the vaccinia P7.5 promoter.

In one embodiment, the mammal is human.

In further embodiments, the above methods can be used to treat melanoma in a patient in need of such treatment.

The invention further provides compositions and kits comprising at least 4 mg of a DNA plasmid expressing a melanoma epitope and at least 1 x 10^9 pfu of a replication-deficient poxvirus expressing the same melanoma epitope for use in the methods of the above embodiments, and further provides a DNA plasmid expressing a melanoma epitope and a replication-deficient poxvirus expressing the same melanoma epitope for use in the manufacture of said compositions and kits.

Immunosurveillance for suppression of metastases:

Without being bound by theory, the inventors consider that the heterologous prime-boost immunization against melanoma disclosed herein creates a systemic central memory T

cell immune response which can inhibit the growth of melanoma metastases and inhibit new metastases from forming. Therefore this prime-boost strategy could be advantageously used in earlier stage melanoma patients to inhibit disease progression e.g. after resection of a primary tumour.

This invention therefore also provides a method of suppressing metastasis of melanoma in a mammal, which comprises the steps of:

a) administering to the mammal a priming composition comprising a source of a melanoma epitope

b) administering a boosting composition to the mammal comprising a replication-deficient poxvirus expressing the same melanoma epitope, wherein the priming composition and the boosting composition are different.

In one embodiment, the priming composition is a DNA plasmid expressing the melanoma epitope.

In a preferred embodiment, the melanoma epitope is a CD8+ T cell epitope.

In a preferred embodiment the DNA plasmid expresses the amino acid sequence of SEQ ID No. 2

In a more preferred embodiment, the replication-deficient poxvirus expresses the amino acid sequence of SEQ ID No. 2

In one embodiment the replication-deficient poxvirus is MVA

In one embodiment the boosting composition comprises at least 1×10^9 pfu of replication-deficient poxvirus

In one embodiment the priming composition comprises at least 4 mg of DNA

In specific embodiments the priming and/or boosting compositions are administered in two or more separate doses.

The invention further provides compositions and kits comprising a source of a melanoma epitope and a replication-deficient poxvirus expressing the same melanoma epitope for use in the methods of the above embodiments, and further provides a source of a melanoma epitope and a replication-deficient poxvirus expressing the same melanoma epitope for use in the manufacture of said compositions and kits.

Immunomonitoring to determine disease prognosis and further treatment

In the present invention, the inventors have determined by immunomonitoring during and after the treatment period that an immune response above a patient's baseline level is well correlated with improved survival. Immunological assays used for immunomonitoring include determination of the proportion of epitope-specific CD8+ T cells in peripheral blood by tetramer staining; determination of interferon-gamma-secreting CD8+ T cells in peripheral blood by the ELISPOT assay; determination of circulating memory T cells capable of expansion by culturing peripheral blood lymphocytes and detecting expanded populations by e.g. tetramer or ELISPOT assays; and determination of the phenotype (naïve, effector, effector-memory, central memory) of CD8+ T cells by analysis of cell surface markers CD45RA and CD27.

Therefore the invention also provides a method of determining the prognosis of a melanoma patient receiving immunotherapy, comprising the steps of:

a) conducting an immunological assay on the patient before commencing immunotherapy

b) repeating the immunological assay during or after the immunotherapy

c) correlating an improvement in the assay result of b) compared to the assay result of a) with an improved prognosis for the patient.

In one embodiment the immunological assay is the determination of tetramer positive CD8+ T cells.

In one embodiment the immunological assay is the determination of interferon-gamma secreting CD8+ T cells.

In one embodiment the immunological assay is carried out after in vitro stimulation, for the determination of central memory T cells.

In one embodiment the immunological assay is the phenotypic analysis of CD8+ T cells.

In one embodiment the immunological assay is the determination of change in phenotype from naïve CD8+ T cells towards effector-memory CD8+ T cells and/or effector CD8+ T cells.

In a preferred embodiment the immunotherapy comprises heterologous prime-boost vaccination.

In a particularly preferred embodiment the heterologous prime-boost vaccination comprises administering a DNA plasmid expressing a melanoma CD8+ T cell epitope followed by a recombinant replication-deficient poxvirus expressing said melanoma epitope, and the immunological assay detects CD8+ T cells specific for said melanoma epitope.

In another particularly preferred embodiment the melanoma epitope is a Melan-A epitope.

In a specific embodiment the melanoma epitope is ELAGIGILTV.

In one embodiment, the method further comprises the step of

d) continuing immunotherapy if there is an improvement in the assay result of b) compared to the assay result of a) or commencing an alternative therapy if there is no improvement in the assay result of b) compared to the assay result of a).

In a preferred embodiment continuing immunotherapy comprises administering an additional increased dose of the recombinant replication-deficient poxvirus.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the melanoma CTL poly-epitope gene.

Figure 2 is a list of the amino acid sequences of the melanoma and influenza CTL epitopes encoded by the melanoma CTL poly-epitope gene.

Figure 3 is a schematic of the construction of plasmid pTH.

Figure 4 is a map of the pSG2 plasmid.

Figure 5 is a map of plasmid pSG2.Mel3.

Figure 6 shows the nucleotide sequence (SEQ ID NO: 1) of the assembled CTL poly-epitope gene cassette and predicted amino acid sequence (SEQ ID NO: 2).

Figures 7A-7B show the nucleotide sequence (SEQ ID NO: 3) of plasmid pSG2.Mel3.

Figure 8 is a gel showing the restriction enzyme analysis of plasmids pSG2 and pSG2.Mel3.

Figure 9 show the oligonucleotides used to construct the melanoma CTL poly-epitope gene.

Figure 10 show the oligonucleotide primers used for sequencing the poly-epitope gene in plasmid pSG2.Mel3.

Figure 11 show the oligonucleotide primers used for sequencing of the melanoma poly-epitope gene in MVA.Mel3.

Figure 12 shows the map of plasmid pSC11.Mel3.

Figure 13 is a gel showing the restriction enzyme analysis of plasmids pSC11.Mel3 and pSC11*.

Figure 14 shows the nucleotide sequence of the melanoma poly-epitope gene in recombinant MVA.Mel3 and predicted amino acid sequence (SEQ ID NO: 4).

Figure 15 shows the patient baseline characteristics from Example 4.

Figure 16 shows a safety summary from Example 4.

Figure 17 shows treatment related adverse events by CTC grade and dose from Example 4.

Figure 18 shows injection site reactions at 7 days post immunisation from Example 4.

Figure 19 is a summary of treatment responses.

Figure 20 is a graph of the Melan A specific response in ELISPOT and Tetramer analysis from patient 033.

Figure 21 is a graph of the Melan A specific response (ELISPOT and Tetramer) from patient 047.

Figure 22 is a graph of the ELISPOT assay with epitopes except Melan A from week 0-16 from patient 047.

Figure 23 show data from the RECIST Tumour Assessment ("Best Overall Tumour Response after at least 16 Weeks "Partial Response - Patient 033").

Figure 24 is a graph of an ELISPOT analysis of Patient 033 from all epitopes weeks 0-16.

Figure 25 is a graph of an ELISPOT analysis of Patient 033 from all epitopes weeks 16-33.

Figure 26 is a graph of an ELISPOT analysis of Patient 047 from all epitopes weeks 0-16.

Figure 27 shows the average fold increases in ELISPOT and Tetramer immune response from groups 1-7

Figure 28 shows titres of anti-MVA antibodies before and after immunization

Figure 29 shows fold increases of anti-MVA antibodies

Figure 30 is a Kaplan-Meier survival chart comparing ELISPOT responders with non-responders.

Figure 31 is a Kaplan-Meier survival chart comparing Tetramer responders with non-responders.

Figure 32 shows ELISPOT responses in patients who received additional boosting vaccinations

Figure 33 shows ELISPOT responses in patients who received additional boosting vaccinations

Figure 34 shows ELISPOT, tetramer and phenotype analysis for patient 033. The ELISPOT and tetramer results continue on from Figure 20.

Figure 35 shows tetramer (A) and T cell phenotype (B) analysis for patient 047

Figure 36 shows tetramer (A) and T cell phenotype (B) analysis for patient 074

Figure 37 shows a comparison of ex vivo tetramer (A) and ELISPOT (C) with in-vitro stimulated tetramer (B) and ELISPOT (D) results from patient 016

Figure 38 shows a comparison of ex vivo tetramer (A) and ELISPOT (C) with in-vitro stimulated tetramer (B) and ELISPOT (D) results from patient 075

The present invention is based, in part, on the discovery that a boost after an initial boost in a prime-boost regimen significantly potentiates immunological and clinical responses in the individual.

Accordingly, the present invention is directed to a "prime-boost-boost", administration regime, and involves the administration of at least three compositions:

(a) a first composition (priming composition) comprising a source of one or more epitopes of the target antigen;

(b) a second composition (first boosting composition) comprising a source of one or more epitopes of the target antigen, including at least one epitope which is the same as an epitope of the first composition; and

(c) a third composition (second boosting composition) which is the same as the boosting composition of (b) except that this boosting composition is administered at a dose that is higher than the dose of the boosting composition of (b).

The methods and compositions of the present invention can be used to induce a "de novo" immune response against an (one or more) antigen. Alternatively, the methods and compositions of the present invention can be used to boost a pre-existing immune response against an antigen.

As used herein, "mammal" and "mammalian" refer to any vertebrate animal, including monotremes, marsupials and placental, that suckle their young and either give birth to living young (eutharian or placental mammals) or are egg-laying (metatharian or nonplacental mammals). Examples of mammalian species include humans and primates (e.g., monkeys, chimpanzees), rodents (e.g., rats, mice, guinea pigs), ruminents (e.g., cows, pigs, horses), canines and felines.

The methods and compositions described herein can induce, for example, a T cell immune response (e.g., CD8+ T cell, a CD4+ T cell) and/or a humoral (antibody) immune response.

T cells fall into two major groups which are distinguishable by their expression of either the CD4 or CD8 co-receptor molecules. CD8-expressing T cells are also known as cytotoxic T cells by virtue of their capacity to kill infected cells or tumour cells. CD4-expressing T cells, on the other hand, have been implicated in mainly "helping" or "inducing" immune responses.

The nature of a T cell immune response can be characterised by virtue of the expression of cell surface markers on the cells. T cells in general can be detected by the present of TCR, CD3, CD2, CD28, CD5 or CD7 (human only). CD4+ T cells and CD8+ T cells can be distinguished by their co-receptor expression (for example, by using anti-CD4 or anti-CD8 monoclonal antibodies, as is described in the Examples).

Since CD4+ T cells recognise antigens when presented by MHC class II molecules, and CD8+ recognise antigens when presented by MHC class I molecules, CD4+ and CD8+ T cells can also be distinguished on the basis of the antigen presenting cells with which they will react.

Within a particular target antigen, there may be one or more CD4+ T cell epitopes and one or more CD8+ T cell epitopes. If the particular epitope has already been characterised, this can be used to distinguish between the two subtypes of T cell, for example on the basis of specific stimulation of the T cell subset which recognises the particular epitope.

The induction of a CD4+ or CD8+ immune response will cause an increase in the number of the relevant T cell type. This may be detected by monitoring the number of cells, or a shift in the overall cell population to reflect an increasing proportion of CD4+ or CD8+ T cells). The number of cells of a particular type may be monitored directly (for example by staining using an anti-CD4+/CD8+ antibody, and then analysing by fluorescence activated cell scanning (FACScan)) or indirectly by monitoring the production of, for example a characteristic cytokine. CD4+ and CD8+ T cell responses are readily distinguished in ELISPOT assays by specific depletion of one or other T cell subset using appropriate antibodies. CD4+ and CD8+ T cell responses are also readily distinguished by FACS (fluorescence activated cell sorter) analysis.

The methods comprise administering one or more epitopes (e.g., CD8+ T cell epitopes, CD4+ T cell epitopes) of a (one or more) target antigen. Compositions of the invention may comprise one or more epitopes (e.g., CD8+ T cell epitopes, CD4+ T cell epitopes) of one or more target antigen.

In a particular embodiment, the epitope is a T cell epitope. A T cell epitope is a short peptide derivable from a protein antigen. Antigen presenting cells can internalise antigen and process it into short fragments which are capable of binding MHC molecules.

The specificity of peptide binding to the MHC depends on specific interactions between the peptide and the peptide-binding groove of the particular MHC molecule.

Peptides which bind to MHC class I molecules (and are recognised by CD8+ T cells) are usually between 6 and 12, more usually between 8 and 10 amino acids in length. The amino-terminal amine group of the peptide makes contact with an invariant site at one end of the peptide groove, and the carboxylate group at the carboxy terminus binds to an invariant site at the other end of the groove. The peptide lies in an extended confirmation along the groove with further contacts between main-chain atoms and conserved amino acid side chains that line the groove. Variations in peptide length are accomodated by a kinking in the peptide backbone, often at proline or glycine residues.

Peptides which bind to MHC class II molecules are usually at least 10 amino acids, more usually at least 13 amino acids in length, and can be much longer. These peptides lie in an extended confirmation along the MHC II peptide-binding groove which is open at both ends. The peptide is held in place mainly by main-chain atom contacts with conserved residues that line the peptide-binding groove.

For a given antigen, CD4+ and CD8+ epitopes may be characterised by a number of methods known in the art. When peptides are purified from cells, their bound peptides co-purify with them. The peptides can then by eluted from the MHC molecules by denaturing the complex in acid, releasing the bound peptide, which can be purified (for example by HPLC) and perhaps sequenced.

Peptide binding to many MHC class I and II molecules has been analysed by elution of bound peptides and by X-ray crystallography. From the sequence of a target antigen, it is possible to predict, to a degree, where the Class I and Class II peptides may lie. This is particularly possible for MHC class I peptides, because peptides that bind to a given allelic variant of an MHC class I molecule have the same or very similar amino acid residues at two or three specific positions along the peptide sequence, known as anchor residues.

Also, it is possible to elucidate CD4+ and CD8+ epitopes using overlapping peptide libraries which span the length of the target antigen. By testing the capacity of such a library to stimulate CD4+ or CD8+ T cells, one can determine the which peptides are capable of acting as T cell epitopes.

The epitopes either present in, or encoded by the compositions, may be provided in a variety of different forms; such as a recombinant string of one or two or more epitopes, or in the context of the native target antigen, or a combination of both of these. Epitopes (e.g., CD4+ and CD8+ T cell epitopes) have been identified and can be found in the literature, for many different diseases. It is possible to design epitope strings to generate an immune response against any chosen antigen that contains such epitopes. Advantageously, the epitopes in a string of multiple epitopes are linked together without intervening sequences so that unnecessary nucleic acid and/or amino acid material is avoided. In addition to the epitopes from the target antigen, it may be preferable to include one or more other epitopes recognised by T helper cells or B cells, to augment the immune response generated by the epitope string. Particularly suitable T helper cell epitopes are ones which are active in individuals of different HLA types, for example T helper epitopes from tetanus (against which most individuals will already be primed).

The source of epitopes in the priming or boosting composition in the method according to the invention can be any suitable vehicle which can be used to deliver and/or express one or more epitopes of the target antigen in a mammal. For example, the source of epitopes in the priming or boosting composition in the method according to the invention can be a non-viral vector or a viral vector (e.g., a replicating viral vector, a non-replicating or replication-impaired viral vector).

There is no reason why the first and second compositions should not be identical in that they may both contain the source of epitopes. A single formulation which can be used as a primer composition and as the booster compositions can simplify administration.

However, in a particular embodiment, a heterologous prime-boost regimen (heterologous prime-boost-boost) is used to minimize cross reactivity between the source of epitopes used for the priming composition and the source of epitopes used for the boosting composition (see U.S. Patent No. 6,663,871B1 and Published U.S. Application No. 2003/0138454 which are incorporated herein by reference). In this embodiment, the source of epitopes in the priming compositions is different (heterologous) from the source of epitopes in the boosting composition or boosting compositions (e.g. the first and/or the second boosting composition). For example, in one embodiment, the source of epitopes in the priming composition is not a poxvirus vector, particularly when the boosting

composition is a poxvirus vector, so that there is minimal cross-reactivity between the priming and boosting compositions.

Alternative preferred viral vectors for use in the priming and boosting compositions according to the invention include a variety of different viruses, genetically disabled so as to be non-replicating or replication-impaired. Such viruses include for example non-replicating adenoviruses such as El deletion mutants. Genetic disabling of viruses to produce non-replicating or replication-impaired vectors is well known.

Other suitable viral vectors for use in the priming and boosting compositions are vectors based on herpes virus and Venezuelan equine encephalitis virus (VEE). Suitable bacterial vectors for the priming composition include recombinant BCG and recombinant Salmonella and Salmonella transformed with plasmid DNA (Darji A et al 1997 Cell 91: 765-775).

Alternative suitable non-viral vectors for use in the priming and boosting compositions include lipid-tailed peptides known as lipopeptides, peptides fused to carrier proteins such as KLH either as fusion proteins or by chemical linkage, whole antigens with adjuvant, and other similar systems.

In one embodiment of the invention, the source of epitopes in the priming and boosting compositions is a nucleic acid, which may be DNA or RNA, in particular a recombinant DNA plasmid. The DNA or RNA may be packaged, for example in a lysosome, or it may be in free form.

In another embodiment of the invention, the source of epitopes in the priming and boosting compositions is a peptide, polypeptide, protein, polyprotein or particle comprising two or more epitopes, present in a recombinant string of epitopes or in a target antigen. Polyproteins include two or more proteins which may be the same, or different, linked together. The epitopes in or encoded by the priming or boosting composition are provided in a sequence which does not occur naturally as the expressed product of a gene in the parental organism from which the target antigen may be derived.

In one embodiment, the source of epitopes in the priming composition is a DNA plasmid (e.g., pSG2).

In another embodiment, the source of the epitopes in the boosting composition is a non-replicating or replication impaired recombinant poxvirus vector. In a particular

embodiment, the source of epitopes in the boosting composition is a vaccinia virus vector such as MVA, NYVAC or a strain derived therefrom. Alternatives to vaccinia vectors include avipox vectors such as fowl pox or canarypox vectors. Particularly suitable as an avipox vector is a strain of canarypox known as ALVAC (commercially available as Kanapox), and strains derived therefrom.

The term "non-replicating" or "replication-impaired" as used herein means not capable of replication to any significant extent in the majority of normal mammalian cells or normal human cells. Viruses which are non-replicating or replication-impaired may have become so naturally (i.e. they may be isolated as such from nature) or artificially e.g. by breeding *in vitro* or by genetic manipulation, for example deletion of a gene which is critical for replication. There will generally be one or a few cell types in which the viruses can be grown, such as CEF cells for MVA.

Replication of a virus is generally measured in two ways: 1) DNA synthesis and 2) viral titre. More precisely, the term "nonreplicating or replication-impaired" as used herein and as it applies to poxviruses means viruses which satisfy either or both of the following criteria:

1) exhibit a 1 log (10 fold) reduction in DNA synthesis compared to the Copenhagen strain of vaccinia virus in MRC-5 cells (a human cell line);

2) exhibit a 2 log reduction in viral titre in HELA cells (a human cell line) compared to the Copenhagen strain of vaccinia virus.

Examples of poxviruses which fall within this definition are MVA, NYVAC and avipox viruses, while a virus which falls outside the definition is the attenuated vaccinia strain M7.

Modified vaccinia virus Ankara (MVA) is a strain of vaccinia virus which does not replicate in most cell types, including normal human tissues. MVA was derived by serial passage > 500 times in chick embryo fibroblasts (CEF) of material derived from a pox lesion on a horse in Ankara, Turkey (Mayr et al. Infection (1975) 33: 6-14.). It was shown to be replication-impaired yet able to induce protective immunity against veterinary poxvirus infections. MVA was used as a human vaccine in the final stages of the smallpox eradication campaign, being administered by intracutaneous, subcutaneous and intramuscular routes to > 120,000 subjects in southern Germany. No significant side

effects were recorded, despite the deliberate targeting of vaccination to high risk groups such as those with eczema (Mayr et al. Bakteriol B. (1978)167: 375- 90).

The safety of MVA reflects the avirulence of the virus in animal models, including irradiated mice and following intracranial administration to neonatal mice. The non-replication of MVA has been correlated with the production of proliferative white plaques on chick chorioallantoic membrane, abortive infection of non-avian cells, and the presence of six genomic deletions totalling approximately 30 kb. The avirulence of MVA has been ascribed partially to deletions affecting host range genes K1 L and C7L, although limited viral replication still occurs on human TK-143 cells and African Green Monkey CV-1 cells. Restoration of the K1 L gene only partially restores MVA host range. The host range restriction appears to occur during viral particle maturation, with only immature virions being observed in human HeLa cells on electron microscopy (Sutter et al. 1992). The late block in viral replication does not prevent efficient expression of recombinant genes in MVA.

Poxviruses have evolved strategies for evasion of the host immune response that include the production of secreted proteins that function as soluble receptors for tumour necrosis factor, IL-I β, interferon (IFN)-α/β and IFN-γ, which normally have sequence similarity to the extracellular domain of cellular cytokine receptors (such as chemokine receptors).

These viral receptors generally inhibit or subvert an appropriate host immune response, and their presence is associated with increased pathogenicity. The Il-I β receptor is an exception: its presence diminishes the host febrile response and enhances host survival in the face of infection. MVA lacks functional cytokine receptors for interferon γ, interferon αβ, Tumour Necrosis Factor and CC chemokines, but it does possess the potentially beneficial IL-1 receptor. MVA is the only known strain of vaccinia to possess this cytokine receptor profile, which theoretically renders it safer and more immunogenic than other poxviruses. Another replication impaired and safe strain of vaccinia known as NYVAC is fully described in Tartaglia et al.(Virology 1992, 188: 217-232).

Poxvirus genomes can carry a large amount of heterologous genetic information. Other requirements for viral vectors for use in vaccines include good immunogenicity and

safety. In one embodiment the poxvirus vector may be a fowlpox vector, or derivative thereof.

It will be evident that vaccinia virus strains derived from MVA, or independently developed strains having the features of MVA which make MVA particularly suitable for use in a vaccine, will also be suitable for use in the invention.

MVA containing an inserted string of epitopes (as described in the examples) has been previously described in WO 98/56919.

As noted in the exemplification, strong responses were obtained using a prime-boost-boost immunisation regime particularly when the priming composition and the boosting compositions were from different sources (heterologous) and the second boosting composition was administered at a higher dose than the dose of the first boosting composition. In a particular embodiment, the source of epitopes in the first and second boosting compositions are the same. For example, the source of epitopes in the first and second boosting compositions is MVA. In another embodiment, the source of epitopes in the first and second boosting compositions are different. For example, the source of epitopes in the first boosting composition is MVA and the source of epitopes in the second boosting composition is a replication-impaired fowlpox vector (e.g., ALVAC).

The methods of the present invention can comprise administering one or more (a plurality) doses of the priming composition, followed by one or more doses of the first boosting composition and one or more doses of the second boosting composition. For example, the priming composition and each boosting composition may be administered in two doses.

In a particular immunisation protocol, the second boosting composition is administered at a higher dose than the dose of the first boosting composition. For example, the second boosting composition is administered at a dose that is about 2 times, 3 times, 4 times, 5 times, 6 times, 7 times, 8 times, 9 times, 10 times, 11 times, 12 times, 13 times, 14 times, 15 times, 16 times, 17 times, 18 times, 19 times or 20 times higher than the dose of the first boosting composition. In a preferred embodiment, the second boosting composition is administered at a dose that is about four times greater than the dose of the first boosting composition.

The timing of the individual doses will depend on the individual. For example, the timing of the priming and boosting doses can be in the region of from about one week to three weeks, about 6 weeks to 9 weeks, about 9 weeks to 12 weeks, about 12 weeks to 15 weeks, about 15 to about 18 weeks and about 18 weeks to about 21 weeks apart. In particular embodiments, the timing of the priming and boosting doses can be about 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 week, 11 weeks, 12, weeks, 13 weeks, 14, weeks, 15 weeks, 16 weeks, 17 weeks, 18 weeks, 19 weeks, 20 weeks, 21 weeks, 22 weeks, 23 weeks, 24 weeks or 25 weeks apart.

The target antigen for use in the methods of the present invention can be characteristic of the target disease. If the disease is an infectious disease, caused by an infectious pathogen, then the target antigen may be derivable from the infectious pathogen.

The target antigen may be an antigen which is recognised by the immune system after infection with the disease. Alternatively the antigen may be normally "invisible" to the immune system such that the method induces a non-physiological T cell response. This may be helpful in diseases where the immune response triggered by the disease is not effective (for example does not succeed in clearing the infection) since it may open up another line of attack.

In one embodiment, the antigen may be a tumour antigen, for example gp100, MART-1/Melan A, MAGE (e.g., MAGE-1, MAGE-3), BAGE, GAGE, RAGE, LAGE or NY-ESO.

The antigen may be an autoantigen, for example tyrosinase and tyrosinase-related proteins (e.g., TRP-1, TRP-2).

In another embodiment of the invention, the antigen is derivable from M. tuberculosis. For example, the antigen may be ESAT6 or MPT63.

In another embodiment of the invention, the antigen is derivable from the malaria-associated pathogen P. Falciparum.

The compositions of the present invention may comprise epitopes from more than one antigen. For example, the composition may comprise one or more epitopes from two or more antigens associated with the same disease. The two or more antigens may be derivable from the same pathogenic organism.

Alternatively, the composition may comprise epitopes from a variety of sources. For example, the ME-TRAP insert comprises T cell epitopes from P. falciparum, tetanus toxoid, M. tuberculosis and M. bovis.

The method of the present invention can be used to induce an immune response to a variety of conditions or diseases. In particular, the method of the present invention will be useful in the prevention of diseases such as tuberculosis, HIV, malaria. H. pylori, influenza, hepatitis (e.g., HBV, HCV, CMV, herpes virus-induced diseases (e.g., HSV) , Epstein Barr Virus (EBV), respiratory syncytial virus (RSV) and other viral infections, leprosy, non-malarial protozoan parasites such as toxoplasma, and various malignancies (e.g., prostrate, breast, lung, colorectal, melanoma, renal cancers and/or tumours; virally induced tumours).

The compositions described herein may be employed as therapeutic or prophylactic compositions (e.g., therapeutic compositions, vaccines). Whether prophylactic or therapeutic immunisation is the more appropriate will usually depend upon the nature of the disease. For example, it is anticipated that cancer will be immunised against therapeutically rather than before it has been diagnosed, while anti-malaria vaccines will preferably, though not necessarily be used as a prophylactic.

The methods of the present invention has been demonstrated using melanoma antigens. In one embodiment, the method can be used to induce an immune response against multiple epitopes of melanoma in a human subject. In another embodiment, the immune response against melanoma is a CD8+ T cell response.

There is a clear need for the development of improved therapies for the treatment of melanoma. CD8+ cytotoxic T lymphocytes (CTL) are capable of specifically recognising and destroying tumour cells and the generation of CD8+ T cell responses has therefore been the main focus of efforts aimed at the development of therapeutic anti-melanoma vaccines. Several melanoma antigens have now been isolated and numerous CTL epitopes have been identified. CTL epitope-based vaccine strategies offer a number of potential advantages over whole antigen-based cancer vaccines: the use of a CTL poly-epitope gene approach would allow multiple antigens to be simultaneously targeted, should broaden the patient's spectrum of anti-tumour CTL responses, and minimise the possibility of immune escape. However, a major problem in melanoma immunotherapy has been the identification of a means of inducing a sufficiently strong immune response

in vaccinated individuals. Recent preclinical studies have shown that prime-boost immunisation strategies, for example where DNA vaccination is followed by immunisation with a replicating viral vector, substantially enhance the CTL response obtained against HIV and malaria antigens. In order to test a prime-boost strategy for inducing anti-melanoma CTL responses, recombinant DNA and vaccinia virus constructions are required.

To produce a DNA vaccine vector suitable for use in humans, plasmid pSG2 was constructed and validated by sequencing. This contains an enhancer/promoter/intron cassette for efficient expression of inserted antigens in mammalian cells, a polylinker cloning site, the bovine growth hormone transcription termination sequence, and sequences for propagation and selection in *E. coli*. The use of a kanamycin resistance marker avoids the risk of residual ampicillin-based contaminants in the manufactured product causing problems in sensitive individuals.

Plasmid pSG2.Mel3 was generated by insertion of a poly-epitope gene containing seven melanoma CTL epitopes and an influenza CTL epitope into the BamHI site in the polylinker cloning region of pSG2. As the human melanoma CTL epitopes are not recognised in mice, the influenza CTL epitope was included to enable the immunogenicity of the plasmid to be tested in mice with a Db haplotype. The sequence of the poly-epitope gene was confirmed by sequencing, and both pSG2 and pSG2.Mel3 plasmids were characterised by restriction enzyme analysis. The complete sequence of plasmid pSG2.Mel3 was determined.

Plasmid pSG2.Mel3 contains a poly-epitope gene encoding seven human melanoma CTL epitopes and a mouse influenza CTL epitope under the control of an efficient promoter for expression in mammalian cells. The plasmid also carries sequences for propagation and selection in *E. coli* but is unable to replicate in mammalian cells. As shown herein, pSG2.Mel3 is suitable DNA immunisation vector for use in humans.

Modified vaccinia virus Ankara (MVA) was selected as the vaccinia strain for development of a recombinant virus containing melanoma CTL epitopes. Recombinant MVA is considered to be a promising human vaccine candidate because of its safety profile and immunogenic properties.

A transfer vector (pSC11.Mel3) was generated by insertion of a poly-epitope gene containing seven human melanoma CTL epitopes and a murine influenza CTL epitope between the left and right fragments of the vaccinia thymidine kinase (*TK*) gene present in plasmid pSC11*. As the human melanoma CTL epitopes are not recognised in mice, the influenza CTL epitope was included to enable the immunogenicity of the plasmid to be tested. Plasmid pSC11* also contains the *lacZ* gene between the flanking TK regions. Transfecting chicken embryo fibroblast cells infected with wildtype MVA with pSC11.Mel3 resulted in homologous recombination across the *TK* sequences. Recombinant virus was identified by the presence of blue plaques due to the expression of LacZ. Recombinant virus was plaque-purified 8 times and a stock of MVA.Mel3 was prepared. The virus stock was characterised by titration by both X-gal staining and immunostaining with an anti-MVA antibody. The MVA.Mel3 was also characterised by sequencing the melanoma poly-epitope gene. The sequence obtained was consistent with the predicted sequence.

A novel immunotherapy comprising the DNA plasmid, DNA.Mel3, and the MVA viral vector, MVA.Mel3, containing 7 human HLA-A2 or HLA-A1-restricted CTL epitopes from 5 melanoma antigens (Tyrosine, melan-A/Mart-1, MAGE-1, MAGE-3 NYESO-1) has been demonstrated. The data provided herein was designed to evaluate the safety and immunogenicity of different doses and dosing regimens of a heterologous "PrimeBoost" immunisation schedule comprising DNA.Mel3 "priming" followed by MVA.Mel3 "boosting" in subjects with histologically confirmed Stage III or IV malignant melanoma.

This study evaluates the safety, immunogenicity and clinical response (by RECIST criteria) of increasing doses of DNA plasmid (DNA.Mel3) and MVA viral vector (MVA.Mel3) containing 7 human CTL epitopes from 5 melanoma antigens (Tyrosinase, melan-A, MAGE-1, MAGE-3, NY-ESO-1). The data also indicate that pSG.Mel3 and MVA.Me13 are able to stimulate immunologically non-responsive patients as well as increase pre-existing immune responses.

The present invention is also directed to plasmids and recombinant viral vectors used in the methods described herein. Viruses and nucleic acid molecules, including plasmids and vectors, according to the invention are normally provided in isolated, recombinant and/or purified form. Accordingly, nucleic acid sequences are normally

provided isolated from their natural environment, and may be free or substantially free of other nucleic acid sequences. In one embodiment, the invention is directed to an isolated plasmid comprising the polyepitope string of SEQ ID NO: 2. In another embodiment, the present invention is directed to an isolated recombinant replication-deficient poxvirus (e.g., MVA) comprising the polyepitope string of SEQ ID NO: 2.

The invention in various aspects relates to use of compositions in the manufacture of medicaments. A medicament of the invention may comprise a first boosting composition (or a first composition) and a second boosting composition (or a second composition) as separate components or separate formulations. The medicament may for example comprise the compositions in separate containers such as phials. This can simplify sequential administration. The separate containers may be packaged together or separately.

In another embodiment, the first and second boosting compositions or first and second compositions may form a single component of the medicament or a single formulation, e.g. in the same container such as in a single phial, from which sequential first and second boosting compositions or first and second compositions can be administered.

Where the medicament additionally comprises a priming composition, the priming composition normally forms a separate component of the medicament. Thus, the priming composition is preferably in a separate container (e.g. phial) from the other composition or compositions. The priming composition may be packaged together within the same product as one or more other composition of the medicament, or may be packaged separately.

Additionally, as described elsewhere herein, one or more of the compositions may be the same, or may be different. For example, first and second boosting compositions may be the same, even if present as separate components of a medicament, since it may be preferable to present the compositions in separate containers within a product, in order to simplify sequential administration to an individual.

The priming and boosting compositions used in the method of the invention may conveniently be provided in the form of a "combined preparation" or kit. The priming and boosting compositions may be packaged together or individually for separate sale.

The priming and boosting compositions may be used simultaneously, separately or sequentially for inducing an immune response against a target antigen.

The kit may comprise other components for mixing with one or both of the compositions before administration (such as diluents, carriers, adjuvants etc.- see below).

The kit may also comprise written instructions concerning the vaccination protocol.

The present invention also relates to a product comprising the priming and boosting compositions as defined above. The product may be in the form of a pharmaceutical composition.

The pharmaceutical composition may also comprise, for example, a pharmaceutically acceptable carrier, diluent, excipient or adjuvant. The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice.

In particular, a composition comprising a DNA plasmid vector may comprise granulocyte macrophage-colony stimulating factor (GM-CSF), or a plasmid encoding it, to act as an adjuvant; beneficial effects are seen using GM-CSF in polypeptide form. Adjuvants such as QS21 or SBAS2 (Stoute J A et al. 1997 N Engl J Medicine 226: 86-91) may be used with proteins, peptides or nucleic acids to enhance the induction of T cell responses.

In the pharmaceutical compositions of the present invention, the composition may also be admixed with any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), or solubilising agent(s).

The pharmaceutical composition could be for veterinary (i.e. animal) usage or for human usage.

In general, a therapeutically effective dose or amount of the compositions of the present invention is administered. The dosage for DNA compositions (e.g., DNA priming composition; DNA boosting composition) can be from about 0.5 mg to about 10 mg. In particular embodiments, the dosage for DNA compositions is from about 1 mg to about 4 mg. In a particular embodiment, the dosage for DNA compositions is about 2 mg. The dosage for vector (e.g., viral vector such as MVA) compositions (e.g., vector priming

composition; vector boosting composition) can be from about 1 x 10^7 pfu to about 1 x 10^{10} pfu. In particular embodiments, the dosage for vector compositions is from about 2 x 10^7 to about 5 x 10^9 pfu. In a particular embodiment, the dosage of the vector composition is from about 5 x 10^7 pfu to about 1 x 10^9.

The first boosting composition may be for administration at a dose of about 5 x 10^7 pfu, about 2 x 10^8 pfu, about 5 x 10^8 pfu or about 1 x 10^9 pfu.

As indicated herein, a boosting composition administered after an initial boosting composition (the second boosting composition) in a prime-boost-boost regimen significantly potentiates immunological and clinical responses in the individual. In one embodiment, the dosage of the second boosting composition is about four times greater than the first boosting composition. In a particular embodiment, the dosage of the second boosting composition is from about 2 x 10^8 pfu to about 1 x 10^9 pfu.

The priming and boosting compositions of the present invention can be administered using any suitable route of administration. Tablets or capsules of the agents may be administered singly or two or more at a time, as appropriate. It is also possible to administer the compositions of the present invention in sustained release formulations.

Typically, the physician will determine the actual dosage which will be most suitable for an individual patient and it will vary with the age, weight and response of the particular patient. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

Where appropriate, the pharmaceutical compositions can be administered by inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or

monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

For some applications, preferably the compositions are administered orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents.

For parenteral administration, the compositions are best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood.

For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

The physician will determine the actual dosage which will be most suitable for an individual patient and it will vary with the age, weight and response of the particular patient. It is to be noted that whilst the above-mentioned dosages are exemplary of the average case there can, of course, be individual instances where higher or lower dosage ranges are merited and such dose ranges are within the scope of this invention.

In some applications, generally, in humans, oral administration of the agents of the present invention is the preferred route, being the most convenient and can in some cases avoid disadvantages associated with other routes of administration - such as those associated with intracavernosal (i.c.) administration. In circumstances where the recipient suffers from a swallowing disorder or from impairment of drug absorption after oral administration, the drug may be administered parenterally, e.g. sublingually or buccally.

For veterinary use, the composition of the present invention is typically administered as a suitably acceptable formulation in accordance with normal veterinary practice and the veterinary surgeon will determine the dosing regimen and route of administration which will be most appropriate for a particular animal. However, as with human treatment, it may be possible to administer the composition alone for veterinary treatments.

Example 1 Construction and characterisation of a recombinant plasmid expressing a string of melanoma antigens

Melanoma is a malignant tumour of melanocytes which has increased in incidence in the last twenty years. Although early stage melanoma is highly curable with surgical resection, later stages of melanoma are not curable with current treatments. Despite the availability of both chemotherapeutic and immunomodulatory agents, significant survival advantages have not yet been seen. There is therefore a clear need for the development of improved therapies for the treatment of melanoma. CD8+ cytotoxic T lymphocytes (CTL) are capable of specifically recognising and destroying tumour cells. The generation of CD8+ T cell responses has therefore been the main focus of efforts aimed at the development of therapeutic anti-melanoma vaccines.

Several melanoma antigens have now been isolated and numerous CTL epitopes have been identified. Many of the melanoma antigens that have been identified are tissue-specific differentiation antigens in melanocytes and include gp100, MART-1/MelanA, tyrosinase and tyrosinase-related proteins (TRP)-1 and TRP-2. These antigens are involved in the synthesis of melanin and give both melanocytes and deposits of melanoma tumour their dark pigment. Another group of melanoma antigens are expressed by a diversity of tumour tissues, but are not expressed by normal tissues, other than the testis. These are the so-called "cancer-testis" antigens and are encoded by genes with family names such as *MAGE, BAGE, GAGE, RAGE, and LAGE. NY-ESO-1* also falls into this group and is expressed in a significant proportion of human melanoma cells as well as other tumour cells including breast, ovary, bladder, prostrate and liver.

Melanoma-associated proteins and peptides have been delivered to patients using a variety of novel vaccine technologies, including recombinant viral vectors, DNA immunisation, direct administration with adjuvants, or injection of dendritic cells pulsed with CTL epitopes. These vaccine strategies have so far only achieved moderate success in clinical trials. CTL epitope-based vaccine strategies offer a number of potential advantages over whole antigen-based cancer vaccines. The use of a CTL poly-epitope gene approach would allow multiple antigens to be simultaneously targeted, should broaden the patient's spectrum of anti-tumour CTL responses, and minimise the possibility of immune escape. A variety of strategies might be used to deliver CTL-poly-epitope genes, including recombinant viral vectors, naked DNA or transfected dendritic

cells. Only modest CTL responses have been achieved in patients using conventional homologous boost immunisation regimes and the use of heterologous prime-boost strategies may elicit more potent responses.

Recent preclinical studies have shown that prime-boost immunisation strategies, for example where DNA vaccination is followed by immunisation with a replicating viral vector, substantially enhance the CTL response obtained against HIV and malaria antigens (Hanke et al., Vaccine, 16:439-445 (1998), Schneider et al., Nat. Med., 4:397-402 (1998)). In order to test a prime-boost strategy for inducing anti-melanoma CTL responses, recombinant DNA and vaccinia virus constructions have been made containing a poly-epitope gene encoding seven melanoma CTL epitopes derived from five melanoma antigens. The poly-epitope gene also contains an influenza CTL epitope that is recognised by mice to enable the immunogenicity of the plasmid to be tested prior to human studies. A schematic diagram of the CTL poly-epitope gene is shown in Figure 1. The amino acid sequences of the seven melanoma antigens and the influenza epitope are given in Figure 2. This report describes the construction of a recombinant plasmid containing the melanoma poly-epitope gene.

A DNA plasmid containing a poly-epitope gene encoding seven human melanoma CTL epitopes and a mouse influenza CTL reporter epitope was constructed and characterized.

MATERIALS AND METHODS

Materials and reagents

Buffers and reagents

Buffers and solutions:

Chemical reagents and buffers were purchased from Sigma.

Enzymes and molecular biology reagents:

T4 ligase (Promega M1801)

Restriction endonucleases (NEB)

Sequencing kit (ABI dye terminator)

DNA chromatography columns and buffers (Qiagen GmbH)

Agarose (Sigma A9539)

Site directed mutagenesis kit (Promega GeneEditor Q9280)

Culture reagents:

DH5α competent cells (Gibco 18258-012)

Bacterial growth medium (LB Sigma L7275)

Ampicillin (Sigma A2804)

Kanamycin (Sigma K0879)

DNA reagents:

Oligonucleotides were purchased from MWG Biotech GmbH, Anzinger Strasse 7 D-85560 Ebersberg Germany, or Sigma-Genosys.

Plasmid pRc/CMV was purchased from Invitrogen, PO Box 2312, 9704 CH Groningen, The Netherlands.

Plasmid pUC4K was purchased from Pharmacia, 100 Route, 206 North Peapack, New Jersey 07977, USA.

Plasmid PIC20H, based on pUC19, but with a longer polylinker including BglII as well as BamHI were a gift of Peter Meacock, Genetics Dept, Leicester University (Marsh et al., Gene, 32:481-485 (1984)).

Plasmid pEE14 contains the expression efficient enhancer/promoter/intron A cassette of the human cytomegalovirus (hCMV) strain AD169 (Whittle et al., Protein Eng., 1:499-505 (1987)).

Cells and culture medium:

The bacterial host strain used for DNA manipulation and propagation was Escherichia coli strain DH5α. Cells transformed with plasmid DNA containing the β-lactamase gene were propagated in LB liquid medium containing 50 μg/ml ampicillin or on plates containing the same medium plus 2% (w/v) agar. Cells transformed with plasmid DNA containing the kanamycin resistance marker were propagated in LB liquid medium containing 25 μg/ml kanamycin or on plates containing the same medium plus 2% (w/v) agar.

Experimental Methods

Unless stated otherwise, all DNA manipulations were carried out using standard molecular biology techniques as described in Current Protocols in Molecular Biology, Ed. FM Ausubel, John Wiley & Sons or according to the manufacturers' instructions.

Construction of plasmid pSG2

Plasmid pSG2 was derived from plasmid pTH. To construct plasmid pTH, plasmid pRc/CMV was digested with BamHI and the fragments carrying the ColE1 origin of replication, β-lactamase (for ampicillin resistance), the hCMV promoter and the bovine growth hormone polyadenylation site were gel-purified. These fragments were re-ligated to create plasmid pCMVBam. This plasmid was then partially cut with BamHI, the single-cut DNA was gel-purified, the staggered ends filled in with Klenow polymerase and re-ligated. The resulting plasmid, containing a single BamHI site in the polylinker region was designated pCMV. The enhancer/promoter region of pCMV was then excised using MluI and HinDIII restriction endonucleases and a fragment from plasmid pEE14 containing the enhancer/intermediate early promoter/intron A region of hCMV was ligated between the sites to create plasmid pTH.

To make plasmid pSG2, the ampicillin resistance (Amp-r) marker in pTH was replaced with the kanamycin resistance marker (Kan-r) from the bacterial transposon Tn903 present in plasmid pUC4K. The Kan-r gene was excised from pUC4K on a BspHI fragment and ligated with pTH, also cut with BspHI which released the Amp-r gene.

Subsequently, the plasmid underwent a spontaneous deletion of the sequence following the Kan- r gene. This deletion was found to be stable and did not affect function of the plasmid.

Construction of plasmid pSG2.Mel3

The melanoma poly-epitope string was constructed as a series of three cassettes (A, B and C), each with a BglII site at the 5' end and a BamHI site at the 3' end. Each cassette was formed by annealing a set of four oligonucleotides (e.g., oligonucleotides A1, A2, A3 and A4 for cassette A) and ligating the fragment between the BglII and BamHI sites of plasmid pIC20H. The sequences of the oligonucleotides used to form the cassettes are given in Figure 9.

The sequence of the three cassettes was determined and site-directed mutagenesis (SDM) used to obtain the correct sequence for the B and C cassettes. The three cassettes were joined together to create plasmid Mel3-1 as follows. The plasmid containing the B cassette was linearised with BglII and the BglII-BamHI fragment containing cassette A was inserted. The resulting BglII-BamHI fragment containing the A and B cassettes was excised and inserted at the BglII site in the plasmid containing the C cassette. A single base insertion in cassette A was corrected by SDM. The gene cassette was excised from plasmid Mel3-1 as a BglII-BamHI fragment and inserted into the BamHI site in the polylinker of plasmid pSG2.

Sequencing of the CTL poly-epitope gene

The sequence of the CTL poly-epitope gene was obtained using a sequencing kit purchased from ABI. Primers (THP1 and THP2) were designed according to the expected sequence (see Figure 10). THP1 and THP2 anneal on either side of the cloning site in pSG2 and read the sequence of the insert.

Purification of plasmid DNA

DNA plasmids were propagated in E. coli strain DH5α, purified using anion exchange chromatography columns (Qiagen) and resuspended in water. The

concentration was calculated by spectrophotometric analysis at 260 nm and the DNA was then diluted in PBS.

Sequencing of plasmid pSG2.Mel3

The complete nucleotide sequence of plasmid pSG2.Mel3 was determined by QIAGEN GmbH (Max-Volmer Str 4, 40724 Hilden, Germany).

Restriction enzyme analysis of plasmids pSG2 and pSG2.Mel3

Plasmids pSG2 and pSG2.Mel3 were digested with BamHI/HindIII and PstI restriction enzymes and the resulting fragments were separated on 1.2% (w/v) agarose gels at 100V for 40 minutes. Size markers used were φX174 DNA digested with XhoI and λ DNA digested with HinDIII. The expected size pattern of fragments (base pairs) generated by these digestions are:

Restriction enzymes	pSG2	pSG2.Mel3
BamHI/HindIII	2259, 2097, 18	279, 2259, 2097
PstI	77, 4297	4297, 338

RESULTS

Construction of plasmid pSG2

The construction of plasmid pTH is shown in Figure 3. Plasmid pSG2 was derived from pTH by replacing the ampicillin resistance maker in pTH with a kanamycin resistance marker. A schematic map of plasmid pSG2 is shown in Figure 4.

Construction of plasmid pSG2.Mel3

Plasmid pSG2.Mel3 was constructed by insertion of a BglII-BamHI fragment containing a CTL poly-epitope gene into the BamHI site in the polylinker of plasmid pSG2. Plasmid pSG2.Mel3 contains the hCMV immediate early promoter with intron-A ···

for driving expression of the melanoma-based antigen in mammalian cells, followed by the bovine growth hormone transcription termination sequence. The plasmid also contains the kanamycin resistance gene and is capable of replication in E. coli but not in mammalian cells. A schematic map of plasmid pSG2.Mel3 is shown in Figure 5.

Characterisation of plasmids pSG2 and pSG2.Mel3

Sequence of the CTL poly-epitope gene in plasmid pSG2.Mel3

The DNA sequence and predicted amino acid sequence of the BglII-BamHI fragment containing the CTL poly-epitope gene is shown in Figure 6.

Complete sequence of plasmid pSG2.Mel3

The complete sequence of plasmid pSG2.Mel3 is shown in Figure 7.

Restriction enzyme analysis of plasmids pSG2 and pSG2.Mel3

The DNA fragments generated following digestion of pSG2 and pSG2.Mel3 with restriction enzymes BamHI/HindIII and PstI is shown in Figure 8.

DISCUSSION

Plasmid pSG2 was developed as a novel DNA immunisation vector suitable for use in humans. Firstly, to minimise the size of the vaccine DNA, the neomycin resistance gene and f1 origin of single-stranded DNA replication were removed from plasmid pRc/CMV. Secondly, the pRc/CMV enhancer/promoter region was substituted with an enhancer/promoter/intron cassette for more efficient expression in mammalian cells. Finally, the bacterial β-lactamase gene was replaced with a kanamycin resistance marker in order to avoid the theoretical risk of spreading ampicillin resistance genes to opportunistic bacteria, since ampicillin is still used to treat bacterial infections.

Plasmid pSG2.Mel3 was generated by insertion of a CTL poly-epitope gene containing seven melanoma CTL epitopes and an influenza CTL epitope into the BamHI

site in the polylinker cloning region of pSG2. As the human melanoma CTL epitopes are not recognised in mice, the influenza CTL epitope was included to enable the immunogenicity of the plasmid to be tested in mice with a D^b haplotype. The sequence of the CTL poly-epitope gene was confirmed by sequencing. The complete sequence of plasmid pSG2.Mel3 was determined and the plasmid is 4635 base pairs in size.

The pSG2 and pSG2.Mel3 plasmids were also characterised by restriction enzyme analysis. In all cases, the pattern of fragments generated, and their sizes, were consistent with the predicted pattern based on the sequence of the plasmids.

Plasmid pSG2.Mel3 contains a CTL poly-epitope gene encoding seven human melanoma CTL epitopes and a mouse influenza CTL epitope under the control of an efficient promoter for expression in mammalian cells. The plasmid also carries sequences for propagation and selection in *E. coli* but is unable to replicate in mammalian cells. It is therefore be a suitable DNA immunisation vector for use in humans.

Example 2 Construction and characterisation of recombinant MVA expressing a string of melanoma antigens

As indicated herein, recent preclinical studies have shown that prime-boost immunisation strategies, for example where DNA vaccination is followed by immunisation with a replicating viral vector, substantially enhance the CTL response obtained against HIV and malaria antigens (Hanke et al., Vaccine, 16:439-445 (1998), Schneider et al., Nat. Med., 4:397-402 (1998)). A variety of attenuated recombinant viral vectors have been developed as antigen delivery systems. However, not all attenuated viruses are replication-incompetent in mammalian hosts and the use of attenuated but replication-competent viruses can lead to side effects, particularly in immunocompromised individuals.

Modified vaccinia virus Ankara (MVA) is a strain of vaccinia virus that does not replicate in most cell types, including normal human tissues (Mayr et al, Zentralb. Bakteriol., B., 167:375-390 (1978)). MVA was derived by 576 passages of a vaccinia virus from a horse pox lesion and was administered to 120,000 people in the last stages of the smallpox eradication programme in Germany. The genome of MVA has been fully

sequenced and the virus has six genomic deletions totalling 30kb. The avirulence of MVA has been ascribed in part to deletions of host range genes and it also lacks several genes coding for immunomodulatory proteins. Since infection with replication-impaired viruses is abortive and therefore delivers a lower dose of antigen in vivo, it has been speculated that these viruses would be less immunogenic than their replication-competent parental strains. However, in studies comparing replication-impaired vaccinia viruses with a replication-competent virus, only boosting DNA-primed animals with replication-impaired poxviruses induced high levels of protection against malaria (Schneider et al., Nat. Med., 4:397-402 (1998)). Recombinant MVA is therefore considered to be a promising human vaccine candidate because of its safety profile and immunogenic properties.

In order to test a prime-boost strategy for inducing anti-melanoma CTL responses, recombinant DNA and vaccinia virus constructions have been made containing a poly-epitope gene encoding seven human melanoma CTL epitopes derived from five melanoma antigens. The poly-epitope gene also contains an influenza CTL epitope that is recognised by mice to enable the immunogenicity of the plasmid to be tested prior to human studies.

A recombinant MVA containing a poly-epitope gene encoding seven human melanoma CTL epitopes and a mouse influenza CTL reporter epitope was constructed and characterized.

MATERIALS AND METHODS

Materials and reagents

Buffers and reagents

Buffers and chemicals:

Chemical reagents and buffers were purchased from Sigma

Ethanol was purchased from Riedel de Haen

Enzymes and molecular biology reagents:

T4 ligase (Promega (M1801))

Restriction endonucleases (NEB)

Sequencing kit (ABI dye terminator)

DNA chromatography columns and buffers (Qiagen GmbH)

Agarose (Sigma (A9539))

Bacterial culture reagents:

Bacterial growth medium LB (Sigma (L7275))

Ampicillin (Sigma (A2804))

Kanamycin (Sigma (K0879))

DH5α competent cells (Gibco (18258-012))

Tissue culture reagents:

Foetal calf serum (FCS) (Sigma)

Superfect (Qiagen)

MEM (Sigma)

Penicillin (100 units) (Sigma)

Streptomycin (100 μg/mL) (Sigma)

Tissue culture plates (Falcon)

X-gal (Promega)

Formaldehyde (37%) (Sigma)

Carboxymethoxy cellulose (CMC) (BDH (276494N))

CMC overlay - Prepare 3% CMC in water and autoclave. Mix 1:1 with 2 x MEM containing 4% FCS and 2 x penicillin/streptomycin.

Immunoassay reagents:

96-well nitrocellulose plates (Milliscreen MAHA, Millipore, UK)

24-well plates (Corning Costar)

Bovine serum albumin (BSA) (Sigma)

Fast DAB kit (Sigma (D-0426))

Anti-mouse IgG peroxidase conjugate (Sigma Immunochemicals (A-2554))

Anti-MVA antibody - Mouse serum from BALB/c mice immunised twice with 1 x 10^6 plaque-forming units (pfu) of MVA

DNA and viral reagents

Oligonucleotides were purchased from R&D Systems Europe Ltd, 4-10 The Quadrant, Barton Lane, Abingdon, Oxon OX14 3YS.

Plasmid pSC11* was a gift from Dr E Cerundolo, Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DS.

Non-recombinant MVA was obtained from Anton Mayr, University of Munich Germany.

Cells and culture medium

The bacterial host strain used for DNA manipulation and propagation was Escherichia coli strain DH5α. Cells transformed with plasmid DNA containing the β-lactamase gene were propagated in LB liquid medium containing 50 μg/mL ampicillin or on plates containing the same medium plus 2% (w/v) agar. Cells transformed with plasmid DNA containing the kanamycin resistance marker were propagated in LB liquid medium containing 25 μg/ml kanamycin or on plates containing the same medium plus 2% (w/v) agar.

Recombinant and non-recombinant vaccinia viruses were routinely propagated in primary chicken embryo fibroblasts (CEFs) grown in minimal essential medium (MEM) supplemented with 10% (v/v) foetal calf serum (FCS). For growth, CEFs were cultivated in MEM with 10% (v/v) FCS and incubated at 37°C. For maintenance, CEFs were incubated in MEM with 2% (v/v) FCS and incubated at 30°C.

MVA is unable to infect cells at a FCS concentration of 10% (v/v). For infection, CEFs are therefore grown in MEM with 10% (v/v) FCS, rinsed in phosphate-buffered saline (PBS) and virus added in MEM containing 2% (v/v) FCS.

Experimental Methods

Unless stated otherwise, all DNA manipulations were carried out using standard molecular biology techniques as described in Current Protocols in Molecular Biology, Ed. F. M. Ausubel, John Wiley & Sons or according to the manufacturer's instructions.

Construction of plasmid pSC11.Mel3

Plasmid pSC11 (Chakrabarti et al., Mol. Cell Biol., 5:3403-3409 (1985)) contains the vaccinia late/early P7.5 promoter (Cochran et al., J. Virol., 54:30-37 (1985)) to drive expression of the inserted antigen, and the vaccinia late promoter P11 driving expression of the *lacZ* marker gene. It also contains the left and right fragments of the vaccinia thymidine kinase (*TK*) gene flanking the region containing the *lacZ* gene and the inserted antigen so that these sequences can be inserted into the MVA genome by homologous recombination at the *TK* locus, thereby inactivating the *TK* gene. Plasmid pSC11* with a polylinker (SalI, BglII, ApaI, KpnI, NotI) under the control of the P7.5 promoter is described by Bacik et al., J. Immunol., 152:381-387 (1994).

Plasmid Mel3-1 contains the melanoma CTL poly-epitope gene cassette. To generate plasmid pSC11.Mel3, the poly-epitope cassette was excised from plasmid Mel3-1 as a BglII-BamHI fragment and inserted into the BglII site in the polylinker of plasmid pSC11*.

Purification of plasmid DNA

DNA plasmids were propagated in E. coli strain DH5α, purified using anion exchange chromatography columns (Qiagen) and resuspended in water. The concentration was calculated by spectrophotometric analysis at 260 nm and the DNA was then diluted in PBS.

Restriction enzyme analysis of plasmid pSC11.Mel3

Plasmids pSC11* and pSC11.Mel3 were digested with restriction enzymes BglII/NotI and EcoRI and the resulting fragments were separated on 1.2% (w/v) agarose gel at 100V for 40 minutes. Size markers used were φX174 DNA/HaeIII and λ DNA/HinDIII. The expected size pattern of fragments (base pairs) generated by these digestions were:

Restriction enzymes	pSc11*	pSC11.Mel3
BglII/NotI	7903	7903, 300
EcoRI	4411, 3010, 383, 99	4411, 3010, 683, 99

Selection of MVA.Mel3

Recombinant viruses were produced by infecting primary CEFs with MVA, then transfecting the same cells with the appropriate shuttle vector. CEF cultures (90% confluent) were infected with 1-2 pfu/cell wild type MVA in 1 mL MEM with 2% (v/v) FCS for 120 minutes in a standard tissue culture incubator (37°C, 5% CO_2). After infection, the cells were transfected with pSC11.Mel3 using Superfect. Following a two hour incubation the cells were incubated for 2 days in MEM with 2% (v/v) FCS to allow recombination and viral replication to occur.

Wild type and recombinant viruses were released by repeated freeze/thawing of the cells (3 times in a dry ice/isopropanol bath). The virus mixture was diluted (undiluted, 10^{-1}, 10^{-2} 10^{-3}) in MEM with 2% (v/v) FCS and plated out on fresh CEF monolayers. The monolayers were overlaid with 2 mL of agarose (2% (w/v) low melting point agarose mixed 1:1 with 2 x MEM containing 4% (v/v) FCS and 2 x penicillin/streptomycin).

Following 48 hours incubation a further overlay of agarose containing X-gal (0.25 µg/mL) was added. After overnight incubation blue-stained areas containing recombinant virus expressing LacZ could be identified. These were isolated by picking areas containing agarose and the underlying infected cells with Gilson P1000 pipette tips. Virus was released by freeze thawing (3 times), diluted, sonicated and re-plated as described. This procedure was repeated eight times.

After 8 rounds of plaque purification, a T-25 flask of CEFs (1.5×10^6 cells) was infected with 0.1 mL of the plaque-purified material. A cytopathic effect (CPE) was observed after 2-3 days and the cells were then harvested. Titrations were done in quadruplicate using two methods; staining with X-gal to detect recombinant MVA and immunostaining to detect all MVA. The virus was amplified further by infecting CEFs at a multiplicity of infection of one.

Preparation of MVA virus stocks

Bulk stocks of recombinant MVA were grown on primary CEFs. Ten flasks (T-150 flasks with CEFs almost confluent) were infected with 1×10^8 pfu (total for 10 flasks). Eight mL MEM with 2% (v/v) FCS was used per flask. Following 2 hours incubation, 20 mL MEM with 2% (v/v) FCS was added. Maximum CPE was visible after 2-3 days. Cells were scraped into the medium and pelleted in 50 mL Falcon tubes. Care was taken not to lyse the cells at this stage in order to minimise the loss of virus into the medium. The cells were transferred to a 15 mL tube and residual medium was removed. The cell pellets were resuspended in 2 mL Tris buffer (10 mM Tris, pH 9.0), freeze/thawed 3 times (freeze in dry ice/ethanol bath, thaw in 37°C water bath) and sonicated for 2 minutes in a cup horn attached to a sonicator.

The resuspended material was pelleted in a bench centrifuge at 500 rpm for 5 minutes. The supernatants were transferred to a clean tube on ice and 2 mL Tris buffer added to the pellet. The pellets were vortexed and centrifuged again. The supernatants were removed and combined with the first supernatants. Four ultracentrifuge tubes (Beckman 362305) were prepared containing 2 mL 36% (w/v) sucrose in sterile Tris buffer. The virus preparation was slowly layered on top of the sucrose cushions using a Gilson P1000 pipette tip. The tubes were centrifuged in a Beckman Optima TL bench top

ultracentrifuge for 80 minutes at 13,000 rpm, 4°C. The supernatants were removed and the pellets resuspended in a total of 1 mL PBS. Virus content was determined by titration. Virus stocks were stored at -70°C.

Titration of the virus stock

CEFs were plated into 24-well plates (4 x 10^5 cells per well) and incubated overnight to obtain the required confluency. The virus stock was diluted (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}) in MEM with 2% (v/v) FCS and 100 µL aliquots were distributed into 4 wells. Following incubation for 1 hour at 37°C each well was overlaid with approximately 0.4 mL CMC overlay. Plates were incubated for 48 hours.

X-gal staining to detect recombinant MVA

Wells were filled with 1% (v/v) formaldehyde and the cells were fixed for 5 minutes. All liquid was then removed and the wells were washed with PBS (1 mL/well).

For one plate, the following staining solution was prepared (in this order) and 0.4 mL added to each well:

9.73mL H_2O

0.1mL 0.5M $K_3Fe(CN)_6$

0.1mL 0.5M $K_4Fe(CN)_6.3H_2O$

0.02mL 1M $MgCl_2$

0.05mL 50 mg/mL X-gal in dimethylformamide

Blue spots developed after 1 hour at room temperature. The spots were counted in wells where they were well separated, taking the average of the 4 wells prepared from the same dilution. The titre was calculated as follows:

Average number of spots x dilution factor x 10 = pfu/mL

Immunostaining to detect all MVA

The CMC overlay and MEM were carefully removed from the wells and the cells fixed for 5 minutes with 1 mL acetone/methanol (1:1, pre-chilled to -20°C). The wells were then washed with 1 mL of PBS. Murine anti-MVA antiserum was diluted (dilution factor 1/500) in PBS containing 3% (w/v) bovine serum albumin (BSA) and 200 µL added per well. The plates were incubated at room temperature for 1 hour and then the wells were washed twice with PBS.

Anti-mouse IgG peroxidase conjugate was added to the wells (200 µL of a 1 in 1000 dilution in PBS containing 3% (w/v) BSA) and the plates incubated for 1 hour at room temperature. The wells were washed twice with PBS. Substrate solution made up from Sigma Fast DAB kit was added until spots developed (less than 1 hour). The substrate was then removed and the plates were washed with water and allowed to dry. The spots were counted in wells where they were well separated, taking the average of the 4 wells prepared from the same dilution. The titre was calculated as follows:

Average number of spots x dilution factor x 10 = pfu/mL

Sequencing of inserted DNA in MVA.Mel3

The melanoma CTL poly-epitope sequence inserted at the TK locus of MVA was obtained using a sequencing kit purchased from ABI. Primers were designed according to the published sequence. The oligonucleotides used are listed in Figure 11.

RESULTS

Construction and characterisation of plasmid pSC11.Mel3

Plasmid pSC11.Mel3 was constructed by insertion of a melanoma CTL poly-epitope gene BglII-BamHI fragment into the BglII restriction endonuclease site of plasmid pSC11* (Figure 12). Plasmid pSC11.Mel3 therefore contains the vaccinia late/early P7.5 promoter driving expression of the inserted melanoma-derived antigen,

and the vaccinia late promoter P11 driving expression of the *lacZ* marker gene, flanked by the left and right fragments of the vaccinia thymidine kinase (*TK*) gene.

The DNA fragments generated following digestion of pSC11.Mel3 and pSC11* with restriction enzymes BglII/NotI and EcoRI is shown in Figure 13.

Construction and characterisation of MVA.Mel3

Selection and purification of recombinant MVA.Mel3

A recombinant MVA.Mel3 virus was isolated following 8 rounds of plaque purification by infection/transfection of CEF cells with wild type MVA and pSC11.Mel3. A stock of virus was purified by sucrose density centrifugation.

Titration of virus stock

MVA.Mel3 was amplified and purified through a sucrose cushion. This material was titrated using X-gal staining to detect recombinant viruses. The material was also stained using an antiserum that recognises MVA proteins. This immunostaining detects all MVA virus particles (recombinant and non-recombinant). The titres obtained were as follows:

X-gal staining: 1.5×10^8 pfu/mL

Immunostaining: 1.5×10^8 pfu/mL

Sequencing of the inserted CTL poly-epitope gene

The sequence of the inserted gene in MVA.Mel3 and the predicted amino acid sequence are given in Figure 14.

DISCUSSION

The transfer vector pSC11.Mel3 was generated by insertion of a poly-epitope gene encoding seven human melanoma CTL epitopes and a murine influenza CTL epitope

between the left and right fragments of the vaccinia thymidine kinase (TK) gene present in plasmid pSC11*. As the human melanoma CTL epitopes are not recognised in mice, the influenza CTL epitope was included to enable the immunogenicity of the plasmid to be tested in mice with a D^b haplotype. Plasmid pSC11* also contains the lacZ gene between the flanking TK regions. Plasmid pSC11.Mel3 was characterised by restriction enzyme analysis. The pattern of fragments generated, and their sizes, were consistent with the predicted pattern based on the sequence of the plasmid.

Transfecting CEF cells infected with wild-type MVA with pSC11.Mel3 resulted in homologous recombination across the TK sequences. Recombinant virus was identified by the presence of blue plaques due to the expression of LacZ. Recombinant virus was plaque-purified eight times and a stock of MVA.Mel3 was prepared.

The virus stock was characterised by titration using X-gal staining and immunostaining with an anti-MVA antibody. The titrations demonstrated an average yield of 1.5×10^8 pfu/mL and confirmed the purity of the recombinant virus.

MVA.Mel3 was also characterised by sequencing the melanoma poly-epitope gene. The sequence obtained was consistent with the predicted sequence.

Recombinant MVA.Mel3 contains a poly-epitope gene encoding seven human melanoma CTL epitopes and a murine influenza CTL epitope under control of the vaccinia P7.5 promoter. It also contains the bacterial lacZ marker gene under the control of the vaccinia P11 promoter. MVA is a strongly attenuated vaccinia virus strain and the recombinant MVA.Mel3 virus is therefore be a suitable immunisation vector for use in humans.

Example 3 Patient Study

MATERIALS AND METHODS

PBMC preparation, storage and shipment

PBMC were isolated from approximately 50 mL of whole blood. Samples were cryopreserved in aliquots of 5 million cells per vial and stored in liquid nitrogen (<150°C). The vials from each sample were shipped overnight on dry-ice (<50°C) in two separate batches. Each shipment contained a temperature monitor to ensure that the

samples had remained at <50°C through out the shipment. On receipt, the samples were transferred to a liquid nitrogen vapour phase storage vessel where they remained until analysis.

Enumeration and quality assessment of PBMC preparations

Cryopreserved PBMC samples were thawed. Cells were enumerated by trypan blue exclusion counting and the cell viability (viable versus total number of cells) was determined. Percentage recovery per vial was determined by comparing the total viable cell count of thawed cells with the number of cells cryopreserved per vial (5 million). Both percentage viability and recovery were recorded as determinates of the PBMC quality. Thawed PBMC preparations showing a viability of <80% were excluded from further analysis to avoid erroneous results. To obtain an estimate of the total number of cells available for the immune analysis, "recovery yield" was determined by multiplication of the "total yield" obtained at the clinical site by the percentage recovery. A recovery yield of approximately 8 million cells was required to conduct a full immune analysis. When less than 8 million cells were available, tetramer analysis was conducted as a priority and ELISPOT analysis was conducted with any remaining cells using a reduced number of replicates.

Tetramer Analysis

The frequency of CD8+ T cells that recognise the Melan-A analogue epitope was determined by tetramer staining. Samples were analysed in triplicate using a Partec PAS FACs machine. All available samples (baseline – week 16) from one patient were analysed in the same experiment and each analysis included isotype, negative and positive controls. The negative control was PBMC isolated from an HLA-A2 negative health volunteer (buffy coat #18) and the positive control was a Melan-A specific short-term T cell line. FACs data were entered into a spreadsheet to calculate the mean ± standard deviation (sd) of the percentage of Melan- A tetramer+ CD8+ T cells in each sample. Since the number of cells in each replicate was not constant and the frequency of MelanA tetramer+ CD8+ T cells was low, mean ± sd were determined according to the Poisson distribution in accordance with the NCCLs guidelines.

ELISPOT Analysis

The frequency of cells that secrete IFNγ in response to the tyrosinase leader, tyrosinase internal, Melan-A analogue, MAGE-3, MAGE-1, and NY-ESO-1 epitopes was determined by the IFN-γ ELISPOT assay. The T cell immune response against media alone and control antigens including Phytohaemagluttinin (PHA), *Mycobacterium tuberculosis* purified protein derivative (PPD) and a pool of MHC Class I restricted peptides from influenza, cytomegalovirus and Epstein Barr virus (FEC) was also evaluated for each sample in the ELISPOT assay. Complete patient profiles (week 0 – 16) were analysed at one time and each epitope peptide or control was analysed in quadruplicate. Where insufficient numbers of cells were available for the analysis, the number of replicates was reduced as appropriate. An independent control sample stimulated with media alone, FEC, PPD, and PHA in duplicate was included on each ELISPOT plate to allow monitoring of inter- and intra-assay variability.

ELISPOT plates were read using an AID ELISPOT plate reader following the appropriate cellular immunology operating procedure. Raw data were cut and pasted into a spreadsheet to calculate the mean±sd number of antigen-specific IFNγ spot-forming cells (SFC) per million PBMC. Since the number of cells in each replicate was constant, the mean ± sd SFC/million was determined according to the normal distribution in accordance with the NCCLs guidelines. Samples exceeding 100 SFC/million PBMC in the media alone wells were excluded from further analysis to avoid the excessive background leading false positive results.

Data Analysis

For the tetramer assay, treatment responders are defined as those in which the mean percentage of Melan-A tetramer+/CD8+ T cells exceeds the mean + 2sd of the baseline response. For the ELISPOT assay, peptide specific-responses are defined as those exceeding the mean + 3sd SFC/million PBMC of the media alone control for the same time point. Treatment specific responses are defined as those peptide-specific responses that significantly increase across the time course.

PROCEDURE

MATERIALS

Chemicals, Reagents and Media

Biotinylated A*0201 / ELAGIGILTV monomer, 0.4 mg/mL [Proimmune; catalogue # MIB-G 082]

Streptavidin R-PE, 1.0 mg/mL [Molecular Probes; catalogue # S-866]

Phosphate-buffered saline (PBS) [Invitrogen; catalogue # 10010-015]

Bovine serum albumin (BSA) [Sigma; catalogue # A-7030]

Sodium Azide (NaN3) [Sigma; catalogue # S-8032]

Equipment

Pipettes and pipette tips

Gloves and lab coat

Ice bucket and wet ice

Suitable light-proof container (ie. amber sterile tube)

METHOD

Preparation of media

a) 0.025% PBS/NaN3 stock:

Add 0.025 g of Sodium Azide (NaN_3) to 100 mL sterile PBS.

b) 20% stock BSA:

Add 2.0 g of bovine serum albumin (BSA) to 10 mL sterile PBS.

Tetramerisation

a) On ice, in the dark, add 225 µL (90 µg) of the biotinylated monomer (0.4 mg/mL) to a suitable sterile light-proof container.

b) Add a total of 150 µL of streptavidin R-PE (1.0 mg/mL) in 5 equal additions of 30 µL. After each addition, mix by pipetting and incubate for

1 hour on ice, in the dark.

c) After the final (fifth) addition and 1 hour incubation, add the following:

- 24 µL of 20% stock BSA

- 81 µL of 0.025% PBS/NaN3 stock

Final total volume = 480 µL

d) Store at 4°C in a light-proof container.

Tetramer staining of ELAGIGILTV-specific CD8+ T cells in human PBMC

This protocol describes a procedure for ELAGIGILTV-tetramer-PE / CD8-FITC staining of non-infectious peripheral blood mononuclear cells (PBMC) and flow cytometric analysis for the quantification of ELAGIGILTV-reactive CD8+ T cells.

ABBREVIATIONS

Abbreviation Term

CIOP Cellular Immunology operating procedure

ELA ELAGIGILTV-tetramer-PE

FACS Fluorescence-assisted cell sorter

FITC Fluorescein isothiocyanate

HI FBS Heat-inactivated foetal bovine serum

ICF Isotype control-FITC

mAb Monoclonal antibody

NC Negative control cells

PBMC Peripheral blood mononuclear cells

PBS Phosphate-buffered saline

PC Positive control cells

PE Phycoerythrin

PROCEDURE

MATERIALS

PBMC samples

Frozen HLA-A2 negative cells (negative control cells)

Frozen ELAGIGILTV-specific cells (positive control cells)

Frozen clinical test samples

Monoclonal antibodies

Anti-human CD8-FITC [Becton Dickinson; catalogue # 551347]

Isotype control-FITC (ICF) [Becton Dickinson; catalogue # 555573]

Chemicals, Reagents and Media

Microsol3 concentrate [Anachem; catalogue # MIC-003]

Sheath fluid, 20L [Partec; catalogue # 04-4008]

Phosphate-buffererd saline (PBS) [Invitrogen; catalogue # 10010-015]

Heat-inactivated foetal bovine serum (HI FBS) [refer to CIOP0001]

Sodium azide [Sigma; catalogue # S-8032]

37% formaldehyde [Sigma; catalogue # F1268]

ELAGIGILTV-tetramer-PE [prepared according to CIOP1009]

Equipment

Gloves and lab coat

Benchtop centrifuge [Beckman GS-6R or equivalent]

Centrifuge microplate carriers

Fridge (4°C)

Vortex

Paper towel

Partec PAS flow cytometer

Partec FACS tubes; 55x12-mm, 3.5ml [Partec; catalogue # 04-2000]

V-bottomed 96-well plate [Sterilin; catalogue # SS242]

Pipettes and pipette tips

100 µm nylon cell strainer (CellTrics) [Partec; catalogue # 04-0041-2318]

CIOP1015 Version 1

Multichannel pipette (50-200 µL range)

Plate sealer [Appleton Woods; catalogue # TC300]

Vacuum filter system, 0.22 µm filter [Appleton Woods; catalogue # BC515]

METHOD

Preparation of media

a) FACS-buffer (PBS, 2% HI FBS/ 0.1% Sodium Azide):

To 500 mL of PBS, add 10 mL HI FBS and 500 mg Sodium Azide. Filter (0.22 µm) and store at 4°C for up to 1 month.

b) Fixation buffer (PBS, 1% formaldehyde):

To 500 mL of PBS add 13.5 mL of 37% formaldehyde. Filter (0.22 µm) and store at 4□C for up to 1 month.

Preparation of PBMC and control cells

a) Thaw vials of PBMC and appropriate test cells from liquid nitrogen storage.

b) Count cells.

c) Centrifuge PBMC and control cells at 1500 rpm (500 g) for 5 min at 4°C.

d) Discard supernatant into a waste disposal tray containing Microsol3 concentrate. Re-suspend positive control cells into a final volume of 350 µL cold (4°C) FACS buffer. Re-suspend negative control cells and clinical test samples into a final volume of 350 µL cold FACS buffer.

Staining of PBMC and control samples

a) Dilute appropriate quantities of each tetramer and antibody in cold FACS buffer in FACS tubes. Store in the dark on ice or at 4□C and use on the same day as dilution.

b) Prepare a control plate by transferring 10 µL per well of negative and positive control cells and test PBMC samples to triplicate wells of a Vbottomed 96-well plate. Place the plate at 4°C until step m.

c) Prepare a test plate by transferring 100 µL per well of negative and positive control cells and test PBMC samples to triplicate wells of a Vbottomed 96-well plate.

d) Wash cells in the test plate by centrifugation at 1500 rpm (500 g) for 7 minutes at 4°C.

e) Flick the supernatant from the test plate into a waste disposal tray containing Microsol3 concentrate. Keep the plate upside down and immediately blot on a paper towel to absorb excess fluid. Re-suspend the cells by vortexing each plate.

f) Add 10 µL of diluted tetramer-PE directly to all the wells in the test plate containing cells. Gently pipette up-and down to ensure adequate mixing of the tetramer and the cells. Replace the pipette tip in between each sample.

g) Incubate cells in the test plate by placing the plate in the fridge (4°C) for 30 minutes.

h) Add 200 µL cold FACS-buffer to each well of the plate using a multichannel pipette and pipette gently up and down to re-suspend cells.

i) Pellet cells by centrifugation of the plate at 1500 rpm (500 g) for 7 minutes at 4°C.

j) Flick the supernatant from the plate into a waste disposal tray containing Microsol3 concentrate. Keeping the plate upside down, immediately blot on a paper towel to absorb excess fluid. Resuspend cells by careful vortexing.

k) Repeat wash and pellet of cells in the test plate once (following steps h to j).

l) Add 10 µL of diluted CD8-FITC antibody directly to the cells in all wells of the test plate. Gently pipette up-and down to ensure adequate mixing of the CD8-FITC mAb and the cells. Replace the pipette tip between each sample.

m) Add 10 µL of diluted ICF antibody directly to the cells in all wells of the control plate. Gently pipette up-and down to ensure adequate mixing of the ICF mAb and the cells. Replace the pipette tip between each sample.

n) Incubate cells by placing both plates in the fridge (4°C) for 30 minutes.

o) Following completion of the incubation, wash and pellet cells in both the control and test plates once (following steps h to j).

p) Add 100 µL cold Fixation-buffer to each well using a multichannel pipette and pipette gently up and down to re-suspend cells. Acquire immediately using a FACS machine (section 6.2.4) or seal plate using a plate sealer and store at 4°C in the dark for up to 18 hours before acquisition.

Preparation of flow cytometer and acquisition of samples.

a) Prior to acquiring clinical samples, the Partec PAS flow cytometer is calibrated for ELAGIGILTV-tetramer-PE / CD8-FITC staining.

b) Prepare flow cytometer.

c) Individually re-suspend each sample in the 96-well plate by gently pipetting up and down with a 100 µL pipette and transfer each sample to a separate FACS tube. Add 0.9 mL of cold Fixation-buffer to each FACS tube and keep the cell suspensions in the fridge or on ice whilst preparing the flow cytometer. If a sample contains cell aggregates, filter through a 100 µm nylon cell strainer.

d) Acquire samples in sequence starting with the negative control cells followed by the test samples and finishing with the positive control cells.

Human IFN-γ ELISPOT Assay for the Analysis of Melanoma-Specific T Cell Responses

PURPOSE

This protocol describes a way to perform an IFN-γ ELISPOT assay for the analysis of melanoma-specific T cell responses in human peripheral blood mononuclear cell (PBMC) samples.

ABBREVIATIONS

Abbreviation Term

BSA Bovine serum albumin

CIOP Cellular Immunology operating procedure

ELISPOT Enzyme-linked immunosorbent spot assay

FEC Influenza/ Epstein-Barr virus/ Cytomegalovirus (Flu/EBV/CMV)

HI FBS Heat-inactivated foetal bovine serum

IFN-γ Interferon-gamma

mAb Monoclonal antibody

PBMC Peripheral blood mononuclear cell

PBS Phosphate-buffered saline

PBS-BSA 1x Phosphate-buffered saline containing 1% (w/v) Bovine serum albumin

PBST 1x Phosphate-buffered saline containing 0.05% (v/v) Tween 20.

PHA Phytohaemagluttinin

PPD Purified Protein Derivative from M. TB

SA-AP Streptavidin-alkaline phosphatase conjugate

SOP Standard operating procedure

PROCEDURE

MATERIALS

Chemicals, Reagents and Media

Human IFN-γ ELISpot kit [Mabtech; catalogue # 3420-21] containing:

- Human IFN-γ capture antibody: mAb 1-D1K (mouse IgG1), 1 mg/mL.

- Biotinylated anti-IFN-γ detection antibody: mAb 7-B6-1 (mouse IgG1), 1 mg/mL.

- Streptavidin-alkaline phosphatase conjugate (SA-AP).

0.05 M carbonate bi-carbonate buffer, pH 9.6 [Sigma; catalogue # C-3041]

Trypan blue solution, 0.4% [Invitrogen; catalogue # 15250-061]

Phosphate-buffered saline, 1X [Invitrogen; catalogue # 10010-015]

Phosphate-buffered saline, 10X [Invitrogen; catalogue # 70011-036]

Sterile RPMI 1640 medium [Invitrogen; catalogue # 31870-074]

200 mM L-glutamine [Invitrogen; catalogue # 25030-024]

Penicillin-streptomycin solution (5000 units/mL penicillin, 5000 µg/mL streptomycin) [Invitrogen; catalogue # 15070-063]

Heat-inactivated foetal bovine serum (HI FBS)

Heat-inactivated normal human sera

AP conjugate substrate kit [Biorad; catalogue # 170-6432] containing

- reagent A

- reagent B

- 25x colour development buffer

Microsol3 concentrate [Anachem; catalogue # MIC-003]

Deionised Water

Bovine Serum Albumin (BSA) [Sigma; catalogue # A-7030]

Tween-20 [Sigma; catalogue # P-1379]

Positive control PBMC sample

Equipment

96 well MAIP Multiscreen plates [Millipore; catalogue # MAIPS4510]

15 mL Falcon tubes [Appleton Woods; catalogue # CC052]

Nescofilm [VWR; catalogue # 235/0414/01 or similar]

Centrifuge [Beckman GS-6R or equivalent]

Sterile laminar downflow cabinet [Heraeus Herasafe or equivalent]

Multichannel pipette

pH testing strips [Sigma; catalogue # P-4536]

Waterbath [Grant or equivalent]

Pasteur pipettes [Appleton Woods; catalogue # KC257]

Haemocytometer [Scientific Laboratory Supplies; catalogue # HAE2112]

37°C, 5% CO_2 incubator

Plate washer [Nunc Immunowash 12, or equivalent]

Pipettes and sterile pipette tips

Pipette aid and sterile disposable pipettes

Sterile reagent reservoirs

METHOD

Preparation of media and reagents

a) Coating buffer:

Prepare 0.05 M carbonate bi-carbonate coating buffer by dissolving the contents of one capsule of buffer concentrate in 100 mL of deionised water (discard casing), check pH is at 9.6 using a pH testing strip. Autoclave (121°C for 20 minutes) and equilibrate to room temperature before use. Store at room temperature.

b) RO media:

To a 500 mL bottle of RPMI-1640 add 5 mL L-glutamine and 5 mL Penicillin-Streptomycin solution. Store at 4°C for up to 1 month.

c) R10 media:

Add 50 mL of heat inactivated foetal bovine serum (HI FBS) to 500 mL of RO (as above). Store at 4°C for up to 1 month.

d) RN10 media:

Add 5 mL of heat inactivated normal human sera to 45 mL of RO (as above).

Store at 4°C for up to 1 month.

e) PBS-BSA:

Weigh out 5.0 g of BSA and add to a 500 mL bottle of sterile 1X PBS. Filter (0.22 μm) and store at 4°C for up to 1 month.

f) PBST:

To a 5 litre container add 500 mL 10X PBS and 4.5 litres of deionised water. Add 2.5 mL of Tween 20 and mix thoroughly. Store at room temperature for no longer than one week.

Preparation of ELISPOT plate

a) Dilute the anti-IFN-γ capture antibody (mAb 1-D1K, 1 mg/mL, green top) to 10 μg/mL in 0.05 M carbonate bi-carbonate buffer, pH 9.6 [1:100 dilution e.g. 55 μL 1-D1K + 5.5 mL of buffer per plate].

b) Using a multichannel pipette add 50 µL of diluted antibody to each well of a MAIP Multiscreen ELISPOT plate. Tap the plate gently on a flat surface to burst any air-bubbles in the wells and ensure that the wells are covered.

c) Incubate the plate on a flat surface at room temperature for 3 to 8 hours, or seal with Nescofilm and incubate at 4°C for a minimum of 8 hours and a maximum of 7 days (plates older than 7 days must be discarded).

Blocking ELISPOT plate membrane

a) Flick off the capture antibody into a waste disposal tray.

b) Using a multichannel pipette wash the plate once with 180 µL/well sterile 1X PBS. Flick off wash into a waste disposal tray.

c) Add 100 µL/well R10 media and incubate on a flat surface at room temperature for 1 to 8 hours, or seal plate with Nescofilm and incubate at 4°C for 8 to 48 hours.

Thawing and preparation of PBMCs

a) Thaw one vial of PBMCs from each time point.

b) Re-suspend cells in 2.5 mL of R10 and count with Trypan blue.

c) Centrifuge cells at 1500 rpm (500 g) for 5 minutes at room temperature and discard the supernatant into a waste disposal tray containing Microsol3 concentrate (or equivalent disinfectant). Re-suspend PBMC in RN10 media to give a final concentration of 2×10^6 cells/mL (use cell count from Trypan enumeration).

Incubation of PBMCs with antigen(s)

a) Remove a plate of pre-prepared melanoma trial antigens.

b) Remove blocking solution from the ELISPOT plate by flicking into a waste disposal tray.

c) Using a multichannel pipette and sterile reagent reservoir add 50 µL of cell suspension from section 6.2.4 to all test wells.

d) Using a multichannel pipette transfer 50 µL of each pre-prepared antigen from the antigen plate to the test plate from left to right as per plate layout.

e) Ensure that the PBMC make an even cell layer on the bottom of the plate by tapping the plate firmly on all four sides using the palm of one hand whilst holding the lid down tight on the plate to prevent samples mixing between wells.

f) Gently move plate to incubator. Incubate on a flat surface at 37°C, 5% CO_2 for 18-20 hours (overnight).

Developing the ELISpot

a) Aspirate off cell suspensions using a plate washer and wash the plate four times with 200 µL/well PBST.

b) Dilute the detecting antibody (mAb 7-B6-1, 1 mg/mL, yellow top) to 1 µg/mL in PBS-BSA [1:1000 dilution – 5 µL antibody + 5 mL buffer per plate]. Add 50 µL of the diluted antibody per well.

c) Tap the plate gently on a flat surface to burst any air-bubbles in the wells. Incubate on a flat surface at room temperature for 2-4 hours.

d) Aspirate off the detecting antibody using a plate washer and wash the plate four times with 200 µL/well PBST.

e) Dilute the SA-AP (white top) 1:1000 in PBS-BSA [5 µL SA-AP + 5 mL buffer per plate]. Add 50 µL of the diluted SA-AP to each well of the ELISPOT plate.

f) Tap the plate gently on a flat surface to burst any air-bubbles in the wells. Incubate on a flat surface at room temperature for 1-2 hours.

g) Aspirate off the SA-AP and wash the plate four times with 200 µL/well PBST.

h) Make up colour development buffer as follows (per plate):

5 mL deionised water

200 µL 25x AP colour development buffer

50 µL reagent A

50 µL reagent B

i) Mix by inversion and pour into a reagent reservoir then using a multichannel pipette add 50 µL colour development buffer per well. Tap the plate gently on a flat surface to burst any air-bubbles in the wells.

j) Incubate on a flat surface at room temperature for exactly 5 minutes.

k) Wash plate thoroughly with tap water and soak in a container of tap water for 10 minutes.

l) Invert plate(s) on dry tissue and allow to air-dry before counting spots using the AID ELISPOT reader.

Example 4 Evaluation of a Novel Heterologous Primeboost Immunotherapy in Stage III/IV Metastatic Melanoma Patients

A novel immunotherapy comprising a DNA plasmid (pSG2.Mel3, also referred to as DNA.Mel3) and a MVA viral vector (MVA.Mel3) containing 7 human HLA-A2 or HLA-A1-restricted CTL epitopes from 5 melanoma antigens (Tyrosine, melan-A/Mart-1, MAGE-1, MAGE-3 NYESO-1) was constructed. The current study was designed to evaluate the safety and immunogenicity of different doses and dosing regimens of a heterologous "PrimeBoost" immunisation schedule comprising DNA.Mel3 "priming" followed by MVA.Mel3 "boosting" in subjects with histologically confirmed Stage III or IV malignant melanoma. Presented herein are interim safety, immunogenicity and tumour response data from this study.

This study evaluates the safety, immunogenicity and clinical response (by RECIST criteria) of increasing doses of DNA plasmid (DNA.Mel3) and MVA viral vector (MVA.Mel3) containing 7 human CTL epitopes from 5 melanoma antigens (Tyrosinase, melan-A, MAGE-1, MAGE-3, NY-ESO-1).

STUDY DESIGN

Group	N	"Prime"DNA.Mel3 (mg)	"Boost"MVA.Mel3 (pfu)
1	8	2 x 2	$2 \times 5 \times 10^7$
2	5	1 x 4	$2 \times 5 \times 10^7$
3	5	2 x 2	$2 \times 2 \times 10^8$
4	7	2 x 2	$2 \times 5 \times 10^8$
5	6	2 x 2	$2 \times 1 \times 10^9$
6*	5	-	$4 \times 1 \times 10^9$
7*	5	1 x 4	$2 \times 1 \times 10^9$
Total	41		

* Treatment ongoing

Eligibility criteria:

- HLA*A2 (+/- HLA*A1)
- No evidence of brain metastasis
- At least one RECIST measurable lesion or at least one clinically measurable skin lesion
- LDH < twice the upper limit of normal
- No prior surgery within 4 weeks
- No prior cancer therapy within 4 weeks
- No previous other malignancy

The following table shows detailed baseline disease status for the entire study.

Baseline Disease Status

			Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Total
	Stage III /M0		1	1	0	1	0	1	0	4 (10%)
Disease Stage/TNM classification*	IV		7	4	5	6	6	4	5	37 (90%)
		M1 (B or C)	0	0	1	1	1	0	1	4 (10%)
		M1A	2	2	1	2	0	0	0	7 (17%)
		M1B	4	2	3	0	2	2	2	15 (36%)
		M1C	1	0	0	3	3	2	2	11 (27%)
Metastatic Site	Skin/soft tissue		2	2	3	2	2	3	2	16 (39%)
	Local LN		3	3	2	0	1	1	1	11 (27%)
	Lung		5	2	5	4	6	4	3	29 (70%)
	Distal LN		4	0	0	3	2	3	3	15 (37%)
	Bone		1	0	0	0	0	0	0	1 (2%)
	Liver		0	0	1	5	2	0	2	10 (24%)
	Other		0	0	1	3	1	2	0	7

									(17%)
Previous treatment	Surgery	8	5	5	7	6	5	5	41 (100%)
	Radiotherapy	1	0	1	2	4	1	1	10 (24%)
	Chemotherapy	2	2	2	4	3	5	3	21 (51%)
	Immuno/hormonal therapy	1	1*	2	3	2	4	2	15 (37%)

* TNM (Tumor, Node, Metastasis) classification by American Joint Committee on Cancer (AJCC):

- M1a Distant skin, subcutaneous tissue, or nodal metastases Normal LDH (Lactate dehydrogenase)
- M1b Lung metastases Normal LDH

M1c All other visceral metastases Normal LDH
or Any distant metastasis Elevated LDH

Dosing Regime:

pSG2.Mel3 is administered by intramuscular injection (IM) at weeks 0 (Groups 2 and 7) or weeks 0 and 3 (for Groups 1,3-6). MVA.Mel3 is administered by intradermal injection (ID) at weeks 3 and 6 (Groups 2 and 7) or weeks 6 and 9 (Groups 1,3-6). In addition subjects with stable disease could receive additional "boosting" injections of MVA.Mel3 at weeks 16 and 24. Two subjects (033 and 071) received additional follow-on boosts 8 weeks apart. All study procedures were conducted in out-patient clinics.

Schedule of Assessments

Study Week (N)	-2	0	3	6	7	8	9	10	13	16	24	32	N+8
Immunisation		D	D	M			M			M	M		
Immunology monitoring	◊	◊	◊	◊	◊		◊	◊	◊	◊	◊	◊	Until progression
RECIST	◘					◘				◘	◘	◘	
Screening													
Treatment													
Progression													
Survival													

Notes:

D = DNA.Mel3; M = MVA.Mel3

Groups 2 and 7 received only 1 DNA.Mel3 prime and received MVA.Mel3 immunizations 3 weeks earlier than shown above.

Immunisations at weeks 16 and 24 were for patients with stable disease only

The patient baseline characteristics are shown in Figure 15; a safety summary is provided in Figure 16; treatment related adverse events by CTC grade and dose are provided in Figure 17; and injection site reactions at 7 days post immunisation are provided in Figure 18.

The safety data indicates that MVA.Mel3 is safe and tolerable up to $1x10^9$ pfu. Local reactions increased in severity with dose and isolated grade 3/4 systemic reactions typical for MVA were seen following MVA.Mel3 at high doses.

Overall in stage III and IV metastatic melanoma patients the toxicities observed were not dose limiting (Figure 17B).

The safety data coming from this trial showed that the prime-boost dosage regimen was safe and well tolerated, even up to doses of 1 x 10^9 pfu MVA.Mel3 (Figures 16-18). Only serious adverse event (syncope) possibly related to therapy was observed, and while the proportion of Grade 3 adverse events to MVA.Mel3 at the injection site increased in a dose-response manner, there were fewer adverse events following the second MVA.Mel3 injection than at the first MVA.Mel3 injection.

Example 5 - Generation of Immune Responses after Heterologous Prime-Boost Immunotherapy:

IMMUNE MONITORING:

Sampling

• Peripheral blood mononuclear cells (PBMC) were isolated and cryo-preserved at each trial site using a standard protocol within 12 hours of phlebotomy. All samples were shipped on dry ice to Oxxon Therapeutics for analysis.

• PBMC samples were excluded from the analysis if they showed <80% viability in the Trypan blue exclusion count, non-specific background staining in the tetramer assay, or non-specific SFC (>50 SFC/million PBMC) in the ELISPOT assay. Subjects from whom the baseline samples had been excluded were considered ineligible for subsequent immune analysis.

• 36/41 patients were immunologically evaluable. Five patients were excluded from analysis due to poor PMBC quality or early study withdrawal.

Tetramer Assay

• The frequency of Melan-A analogue-specific CD8+ T cells in each PBMC sample was determined by FACs analysis after staining with a Melan-A analogue MHC Class I tetramer conjugated to PE and an anti-human CD8 antibody conjugated to FITC.

• The tetramer results are expressed as the percentage of melan-A analogue+ / CD8+ T cells.

• Responses were defined as those that exceeded the mean + 2 x standard deviation of the baseline (Week 0) response.

ELISPOT Assay

• The frequency of cells that secreted IFNγ in response to the Tyrosinase leader (A2), Tyrosinase internal (A2), Melan-A analogue (A2), MAGE-3 (A1and A2), MAGE-1 (A1), and NY-ESO-1 (A2) epitopes in each PBMC sample was determined by *ex-vivo* IFN-γ ELISPOT assay.

• The results are expressed as the number of IFNγ-spot forming cells (SFC) per million PBMC. Epitope specific-responses were defined as those that exceeded the mean + 3 x standard deviation SFC/million PBMC of the media alone control for all samples from the same patient tested in the same assay.

Figure 19 is a summary of immune responses detected between week 0 and 16.

The following table shows the number of immune responses when grouped by the amount of MVA.Mel3 boost. All had been primed by either 2 x 2 mg or 1 x 4 mg pSG2.Mel3 except for the group marked (homologous).

MVA.Mel3 (pfu)	5×10^7	2×10^8	5×10^8	1×10^9	1×10^9 (homologous)	Total
N (%)	n = 13	n = 5	n = 7	n = 11	n = 5	N(%)
Evaluable	10	5	5	10	5	35 (85%)
Tetramer	5 (50%)	3 (60%)	4 (80%)	9 (90%)	2 (40%)	23 (66%)
ELISPOT	3 (30%)	1 (20%)	3 (60%)	3 (30%)	1 (20%)	11 (31%)

Notes:

Tetramer responses were specific for Melan-A

ELISPOT responses were observed to Melan-A (n = 9), NY-ESO-1 (n = 2) and MAGE-3(A1) (n=1)

Multiepitope ELISPOT responses

Patient 047 exhibited a pre-existing response against the immunodominant Melan-A-analogue epitope detected in both the Tetramer and ELISPOT assays that was boosted during the treatment. In addition, responses against the MAGE-3 and NY-ESO-1 epitopes were evident in this patient after the administration of PrimeBoost treatment at weeks 10 and 13 (Figure 26) . Figure 26 is a graph of an ELISPOT analysis of Patient 047 from all epitopes weeks 0-16.

Patient 013 showed a Tetramer response to the Melan-A analogue epitope and an ELISPOT assay response to the NY-ESO-1 epitope, indicating that prime boost treatment had induced a multiepitope response in this patient. Overall, the majority of patients exhibited responses only to the immunodominant Melan-A analogue epitope. However, patients 047 and 013 provide a clear demonstration that the treatment can induce multiepitope responses and it is notable that in patient 013 the response elicited against the NY-ESO-1 epitope was more active than that directed against the Melan-A analogue epitope.

Figure 27 shows the average fold increases in tetramer (A-C) and ELISPOT (D-F) immune responses against the Melan-A epitope for each group. Fold-differences were calculated by normalising each patient's immune response at time = 0 to 1. Arrows indicate time of immunizations (A, D show groups immunized with 2 x pSG2.Mel3, 2 x MVA.Mel3; B, E shown groups immunized with 1 x pSG2.Mel3, 2 x MVA.Mel3; C, F show groups immunized with 4 x MVA.Mel3). Fold increases in both ELISPOT and Tetramer immune responses are clearly observed in DNA-prime/MVA-boost groups with MVA.Mel3 doses of 5×10^8 pfu or higher (groups 4, 5, 7). No average increase of immune response after baseline was observed in group 6 which had homologous immunizations of 10^9 pfu MVA.Mel3

Figure 20 is a graph of the Melan A specific "De Novo" response in ELISPOT and Tetramer analysis from patient 033. Figure 21 is a graph of the Melan A specific boosted response (ELISPOT and Tetramer) from patient 047. Figure 22 is a graph of the ELISPOT assay with epitopes except Melan A from week 0-16 for patient 047. Figure 23 show data from the RECIST Tumour Assessment ("Best Overall Tumour Response after at least 16 Weeks"; "Patients Showing Time to Progression of at least 16 Weeks"; "Partial Response - Patient 033").

Example 6 – Heterologous prime-boost increases clinical benefit vs homologous prime boost and vs control group.

Figure 23 shows a summary of clinical responses. As defined by RECIST criteria, one partial response (tumour regression > 30%) 5 stable disease at 24 weeks and 2 mixed responses at 24 weeks (classed with Stable disease) for a total of 8 beneficial responses were recorded in the heterologous prime-boost groups (n = 36). i.e. 22% of the patients receiving heterologous prime-boost did not progress at 24 weeks, unlike homologous prime-boost (group 6) where 5/5 patients progressed by 24 weeks (Figure 23A). 2 out of 3 stage 3 patients showed clinical responses (PR or SD). Figure 23B shows the clinical progress of patient 033, who is discussed in more detail in Example 9.

The following table shows the median time to progression and survival for heterologous vs homologous prime-boost. Time to progression was measured by RECIST criteria (evaluations at eight-week intervals) and therefore the data presented here may not reflect incremental improvements in TTP.

	Heterologous (Gps 1-5, 7)	**Homologous (Gp 6)**
	N=36	N=5
TTP Median (weeks)	9.5	8.0

Survival		
Median (weeks)	52	26.0

The median survival of patients excluded from the trial due to being HLA-A2 negative was also monitored as a control. The excluded arm, with access to alternative therapies at the physician's choice, had a median survival of 40 weeks.

Therefore, the median survival provided by heterologous prime-boost melanoma immunotherapy exceeds that of homologous prime-boost and alternative available therapies administered to the HLA-A2 negative excluded arm.

Example 7 – Heterologous prime-boost provides increased survival among immune responders.

The correlation between immune responders (Elispot/Tetramer) to Melan-A and median survival has been investigated. Figures 30 and 31 show Kaplan-Meier survival plots based on interim survival data comparing all Elispot responders (Figure 30) and all Tetramer responders (Figure 31) against respective non-responders. Of the 9 Elispot responders, greater than 50% had survived to 93 weeks, compared to a median survival of 50 weeks for non-responders. Similarly, the 23 Tetramer responders which also had a median survival of 93 weeks, compared to only 37 weeks for non-responders.

These data suggest that presence of a detectable immune response within the first 16 weeks is well correlated with improved survival, and that immune monitoring during and shortly after heterologous prime-boost immunizations could be an effective prognosticator of whether to continue immunotherapy or discontinue immunotherapy and switch to alternative therapies.

We have demonstrated that a DNA/MVA PrimeBoost immunotherapeutic approach is safe at the highest dose, is immunogenic and has anti-tumour activity.

Overall this data supports the use of this novel immunotherapeutic at the highest dose (2 x 2 mg or 1 x 4 mg DNA followed by 2 doses of 5 x 10^8 pfu or greater) in melanoma patients with stage III/IV disease.

Without wishing to be bound by theory, while a large effector T cell immune response has been able to reduce tumor mass in some cases, even a smaller T cell response can provide a greatly improved time to progression and median survival among immune responders. This may be due to the ability of the heterologous prime-boost immunotherapy to stimulate immunosurveillance, and thus to suppress and delay metastatic disease, even if not always mounting a high enough response to attack tumour mass directly. Patient 033 provides direct evidence that the immune response elicited by priming with DNA and subsequent boosting with MVA will elicit a response against the tumour

Example 8 – Continued Immunotherapy

During the above-described clinical trial, patients with stable disease or better were eligible for boosting vaccinations. 13 patients received additional boosting vaccinations of MVA.Mel3 at week 16 and 7 patients received additional boosting vaccinations of MVA.Mel3 at week 24. In all but one case, these additional boosts were at the same dosage as the initial respective boosts at week 6 and 9, as is conventional in the art.

Cellular immune responses were evaluated in the 7 patients (014, 016, 021, 031, 033, 071, 075) that received the additional boosting vaccinations at week 24 using Tetramer and ELISPOT assays as described above (Figures 32-33). In the majority of patients, the immune response was determined at week 16, after the administration of the 1st additional booster (week 18 – 22), after the second booster, and at the latest available timepoint. Patients 071 (data not shown) and 033 (Figures 20 and 36) were monitored at additional timepoints.

Patients 021, 031, 033, 071, and 075 exhibited increased Tetramer immune responses (post-study followup or "PSF" responses") after the administration of one or two

additional booster vaccinations. Notably, ELISPOT responses against the Melan-A-analogue epitope that were not evident at week 16 were induced in patients 033 and 075 after the administration of the additional booster vaccinations. Thus, administration of the additional booster vaccinations using MVA.Mel3 clearly had the capacity to induce further immune responses against the encoded melanoma antigens. It is particularly notable that boosting immune responses were induced in patients that had received both low and high dose administrations of MVA.Mel3 and had established humoral antibody responses against the MVA vector (see example 11), indicating that the MVA vector is still effective in inducing a immune response against encoded antigens in the presence of anti-MVA immunity established during prime boost vaccination.

The follow table shows that of the 5/7 patients who showed immune responses to the additional boosts, 4/5 had not progressed to the knowledge of the study whereas 2/2 non-responders eventually progressed. This further supports the ability of prime-boosted immune responses to prevent tumour progression.

Patient	PSF Tetramer Responder?	PSF Elispot responder?	TTP (weeks)	Survival (weeks)
014	No	No	10	82*
016	No	No	49	76*
021	Yes	No	33*	64*
031	Yes	No	27*	69
033	Yes	Yes	73*	91*
071	Yes	No	33*	57*
075	Yes	Yes	24	50*

* = progression or death had not occurred by that timepoint, subsequent data not available

Example 9: Increased dose of boosting vector leading to immune response and partial clinical response.

One patient (patient 033) received a unique immunisation regimen. Patient 033 was a late enrolment into treatment group I, and when this patient was due for additional boosts at week 16, the low-titre MVA.Mel3 used for group I was no longer available. Due to this, patient 033 received a group III MVA.Mel3 dose as a boost instead, at weeks 18 and 25. (The decision to substitute group III boost resulted in a delay from the intended vaccination at week 16, while the vaccination at week 25 was within the +/- 1 week study window for boost vaccinations).

The dose regimen for patient 033 is shown in the following table and his immunological and clinical response is shown in Figure 23 and the following figures Figure 20 and 36 show the Melan A specific response in ELISPOT and Tetramer analysis from patient 033.

Figure 24 is a graph of an ELISPOT analysis of Patient 033 from all epitopes weeks 0-16. Figure 25 is a graph of an ELISPOT analysis of Patient 033 from all epitopes weeks 16-33.

Week	pSG.Mel3 dose (mg)	MVA.Mel3 dose (pfu)
0	2 (prime 1)	-
3	2 (prime 2)	-
6	-	5×10^7 (boost 1)
9	-	5×10^7 (boost 2)
18	-	2×10^8 (boost 3)
25	-	2×10^8 (boost 4)
74		2×10^8 (boost 5)
82		2×10^8 (boost 6)

Patient 033 first showed an anti-Melan-A Elispot response at weeks 7 and 10, then developed elevated responses following the third, MVA.Mel3 boost vaccination, which was at an elevated dose, and developed a further increased immunological response as determined by Elispot shortly after the fourth MVA.Mel3 boost vaccination (Figures 24 and 25). A significant further increase immune responses was observed after boosts 5 and 6,

Patient 033 also qualified as a "partial responder" after the fourth MVA.Mel3 boost. By the end of the study, patient 033's primary tumour measurement had shrunk approximately 50%.

Both the immunological and clinical responses were considered directly attributable to the third and fourth MVA.Mel3 boosts. This is evidence of a dose response within an individual patient, as well as showing that increasing the dose of a viral vector boost after an initial boost in a prime-boost regimen can significantly potentiate immunological and clinical responses.

Patient 033 shows an immune response which appears to be linked to clinical benefit.

Patient 033 shows that increasing the dose of the MVA.Mel3 boost at weeks 18 and 24 increases the magnitude of the immune response and clinical response, and that reboosting increases the magnitude yet again.

Example 10 – Analysis of Immune responses by *In vitro* stimulation and phenotyping of CD8+ T cells

Populations of Melan-A specific CD8+ T cells detected as tetramer responses were further characterised by phenotyping with activation and memory markers in patients 033 (Figure 34), 047 (Figure 35) and 074 (Figure 36). These patients were chosen as they had exhibited substantial tetramer responses during the course of treatment. Notably, patient 047 had a large tetramer response at baseline which was further boosted by treatment, whereas patient 074 had a low baseline tetramer value which expanded significantly during treatment. To conduct phenotyping, patient PBMC samples were stained with Melan-A analogue Tetramer-PE and an anti-CD8-FITC, then co-stained with anti-

CD45RA and anti-CCR7. The percentage of Melan-A analogue Tetramer$^+$/CD8$^+$ cells that expressed CCR7 and CD45RA was determined by FACs analysis.

Based on phenotypic and functional characteristics, antigen-specific T cells have been divided into four classes: naïve, effector, effector memory and central memory.

Phenotypes	Cell Markers
Naïve cells	CD45RA$^+$CCR7$^+$
Effector cells	CD45RA$^+$CCR7$^-$
Effector-Memory cells	CD45RA$^-$CCR7$^-$
Central memory cells	CD45RA$^-$CCR7$^+$

CD45RA$^+$ and CCR7$^+$ naïve T cells are those that have not yet encountered antigen are thought to reside within tissues; CD45RA$^+$ CCR7$^-$ effector T cells are generated within tissues immediately after contact with antigen; CD45RA$^-$ CCR7$^-$ effector memory cells are activated antigen-specific T cells residing within tissues; and CD45RA$^-$ and CCR7$^+$. central memory cells are thought to reside predominantly within lymph nodes and bone marrow. Functionally, effector and effector memory cells are thought reside within tissue and be immediately effective against disease targets, but relatively unable to proliferate and relatively short-lived. In contrast, central memory cells are thought not to exhibit effector functions, but are long-lived and can proliferate rapidly into effector cells after antigen contact, thereby providing a memory immune response.

Interestingly, prime boost treatment elicited or expanded T cell immune responses to the melan-A-analogue epitope in patients 033, 047 and 074 despite the fact that they showed very different phenotypic characteristics at week 0. The Tetramer$^+$ CD8$^+$ cells in the baseline sample from patient 033 were predominantly of the central memory phenotype, whereas those in patient 074 were predominantly of a naïve phenotype. In contrast, patient 047 showed a predominantly effector memory phenotype, which was consistent with the measurable response against melan-A at baseline in the ELISPOT assay. In all cases, prime boost treatment induced the expansion of cells into an effector or effector

memory phenotype; T cells with such phenotype are thought to express effector functions against disease. The phenotypic switch was particularly notable in patient 033 in whom the percentage of tetramer$^+$/CD8$^+$ cells was similar in week 0 and 18 samples, but the phenotype of the cells had switched predominantly from central memory to effector and effector memory.

Despite the expansion of effector and effector memory cells after the administration of prime boost therapy, a small but measurable percentage of central memory T cells was also evident in patients 033 and 074. It is notable that a much smaller central memory population was evident in patient 047 and the patient withdrew from the study at week 13 due to disease progression. Administration of further MVA.Mel3 boosts to patient 033 elicited very substantial expansion the melan-A analogue specific T cell response (figure 20a), providing a further indication of a proliferative memory response in this patient.

Considered together these data indicate prime boost therapy is effective in inducing or expanding immune responses in patients with different immunological baseline characteristics including those that are naïve for the tumour antigen; those that have a quiescent memory response; and those that have an active effector immune response. In addition, the data shows that in the patients who remained in the study after week 16 there was evidence of a sustained central memory immune response.

Further evidence of the induction of a memory immune response was obtained by conducting *in vitro* stimulation (IVS) assays using samples from patients 016 (group 4) and 075 (group 7). To conduct the IVS assays, patient samples were cultured with the 7 melanoma epitope peptides in the presence of IL-2 for 14 days prior to analysis with standard Tetramer and ELISPOT assays. A 14 day culture period in the presence of specific antigens or epitopes is thought to facilitate the expansion of antigen-specific central memory cells; effector and effector memory cells are thought to undergo cell death under these conditions. Thus, this assay provides a sensitive measure of the melanoma-specific central memory cells elicited in the study.

Patients 016 and 075 were chosen on the basis that both continued in the study and had exhibited stable disease. In addition, patient 016 had not shown any evidence of an *ex*

vivo immune response, whereas patient 075 had shown a modest response in both tetramer and ELISPOT assays (see example 8). Following IVS, both patients exhibited substantial Tetramer responses in samples taken after (Figures 37 and 38 B) but not before ((Figures 37 and 38 A) the administration of prime boost treatment. A corresponding ELISPOT response was observed in the IVS samples from patient 016 (Figure 37 C, D), but could not be determined in 075 due to assay failure (Figure 38 C, D). Notably, the response in patient 016 was evident at week 13 after the administration of the second dose of MVA.Mel3 (Fig 37 B) and that in patient 075 was evident at week 4 after the administration of the first dose of MVA.Mel3 (Fig 38 B); this may be the result of the administration of larger doses of pSG2.Mel3 and MVA.Mel3 in the latter patient. Nevertheless, these studies clearly demonstrate that prime boost immunotherapy had elicited a central memory response in both patients.

With wishing to be bound by theory, studies indicate that prime boost therapy will elicit effector immune responses against the melanoma epitopes that in at least one patient correlate with a reduction in tumour mass. Moreover, phenotyping and IVS data also indicate that prime boost immunotherapy establishes a central memory response against the melanoma epitopes, such a response is likely to provide an immunosurveillance function and account for the enhanced survival of patients receiving prime boost immunotherapy.

Example 11 – Anti-MVA seroconversion

Figure 28 shows anti-MVA titres before and after vaccination and Figure 29 shows the fold increase of anti-MVA antibodies after completion of the vaccination schedules. The increase in anti-MVA antibodies is dose-dependent, with 95% of patients given MVA doses $\geq 5x10^8$ PFU (groups 4-7) seroconverting.

Patients that exhibited 4-fold or greater increase in anti-MVA titre during the treatment were defined as seroconverters, indicating that these patients mounted a humoral response against the MVA vector during the treatment. Notably, subjects in groups 1 and 2, which received the lowest titre MVA.Mel3 dose ($5x10^7$ pfu), showed incomplete seroconversion and the lowest fold-increase in anti-MVA antibody titres (5/7 and 0/4; 5.00±3.00 and 1.50±2.56, respectively). In contrast, all subjects in Groups 5, 6 and 7, which received

the highest dose of MVA.Mel3 ($1x10^9$ pfu), seroconverted and showed the highest fold-increase in anti-MVA antibody titres (56.00±40.16; 37.33±24.44; 28.80±23.05; respectively) when compared to other groups. Groups 3 and 4 that received intermediate doses of MVA.Mel3 ($2x10^8$ and $5x10^8$ pfu, respectively) showed similar seroconversion rates (4/5 in Group 3 and 5/6 in Group 4), but the fold-increase in titre was substantially different between these groups. Notably, Group 3 exhibited an average fold-increase of 7.60±5.37, similar to the low dose groups, whereas Group 4 showed a fold-increase of 28.33±28.13 similar the higher dose groups. Therefore, a dose of $1x10^9$ pfu MVA.Mel3 was necessary to elicit seroconversion in 100% of subjects, but a dose of $5x10^8$ pfu MVA.Mel3 appeared to elicit comparable fold-increase in titre across the group.

During assay validation (BioAnaLab) the medium endpoint titre of 18 negative human serum samples tested was 800. Although the trial was not specifically designed to determine whether the subjects exhibited pre-existing immunity to MVA due to smallpox vaccination, the range of anti-MVA antibody titres observed pre-treatment suggests that some of the subjects had a pre-existing antibody response to MVA (Figure 28A). For example, 14 of 36 subjects showed a pre-treatment titre of >800. Interestingly, only 7/14 of these subjects showed a >4-fold increase after treatment and only 8/14 showed a post treatment titre of >12800. Thus, the presence of a high pre-treatment anti-MVA antibody titre did not necessarily lead to a substantial fold-increase in titre or absolute titre after treatment. Also, 5 out of 12 patients that exhibited high baseline titres showed a response in the tetramer assay and 1 of whom also exhibited a response in the ELISpot assay, indicating that a high baseline titre did not inhibit the capacity of the MVA vector to boost a T cell specific response against the Mel3 insert in these patients.

All references cited herein are incorporated by reference in their entirety.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

1. Use of a priming composition and a boosting composition in the manufacture of a medicament for sequential administration to an individual to induce an immune response against an antigen; wherein

the priming composition comprises a source of one or more epitopes of the antigen; and

the boosting composition comprises a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming composition; and wherein

the boosting composition is for sequential administration as a first boosting composition and a second boosting composition, wherein the second boosting composition is for administration at a dose that is higher than the dose of the first boosting composition.

2. Use of a boosting composition in the manufacture of a medicament for boosting a pre-existing immune response to an antigen in an individual to whom a priming composition was previously administered; wherein

the priming composition comprises a source of one or more epitopes of the antigen; and

the boosting composition comprises a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming composition; and wherein

the boosting composition is for sequential administration as a first boosting composition and a second boosting composition, wherein the second boosting composition is for administration at a dose that is higher than the dose of the first boosting composition.

3. Use according to claim 1 or claim 2, wherein the source of epitopes in the priming composition is different from the source of epitopes in the boosting composition.

4. Use of a priming composition, a first boosting composition and a second boosting composition in the manufacture of a medicament for sequential administration to an individual to induce an immune response against an antigen; wherein

the priming composition comprises a source of one or more epitopes of the antigen;

the first boosting composition comprises a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming composition; and

the second boosting composition comprises a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming composition, wherein the source of epitopes of the first boosting composition is different from the source of epitopes of the second boosting composition; and wherein

the second boosting composition is for administration at a dose that is higher than the dose of the first boosting composition.

5. Use of a first boosting composition and a second boosting composition in the manufacture of a medicament for sequential administration to boost a pre-existing immune response to an antigen in an individual to whom a priming composition was previously administered; wherein

the priming composition comprises a source of one or more epitopes of the antigen;

the first boosting composition comprises a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming composition; and

the second boosting composition comprises a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming composition; and wherein

the second boosting composition is for administration at a dose that is higher than the dose of the first boosting composition.

6. Use according to claim 4 or claim 5, wherein the source of epitopes in the priming composition is different from the source of epitopes in the boosting compositions.

7. A composition comprising a priming composition, a first boosting composition and a second boosting composition for sequential administration to an individual to induce an immune response against an antigen; wherein

the priming composition comprises a source of one or more epitopes of the antigen;

the first boosting composition comprises a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming composition; and

the second boosting composition comprises a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming composition, wherein the source of epitopes of the first boosting composition is different from the source of epitopes of the second boosting composition; and wherein

the second boosting composition is for administration at a dose that is higher than the dose of the first boosting composition.

8. A composition according to claim 7, wherein the source of epitopes in the priming composition is different from the source of epitopes in the boosting compositions.

9. Use according to any of claims 1 to 6, or a composition according to claim 7 or claim 8, wherein the priming composition and each boosting composition are for administration in two doses.

10. Use according to any of claims 1 to 6, or a composition according to claim 7 or claim 8, wherein the second boosting composition is for administration at a dose that is about four times greater than the dose of the first boosting composition.

11. A kit for inducing an immune response against an antigen in an individual, which comprises:

a) a priming composition comprising a source of one or more epitopes of the target antigen;

b) a first boosting composition comprising a source of one or more epitopes of the target antigen, including at least one epitope which is the same as an epitope of the priming composition; and

c) a second boosting composition comprising a source of one or more epitopes of the target antigen, including at least one epitope which is the same as an epitope of the priming composition; and

instructions for administering the second boosting composition of c) at a dose that is higher than the dose of the boosting composition of b).

12. A kit for boosting a pre-existing immune response to an antigen in an individual to whom a priming composition comprising a source of one or more epitopes of the antigen was previously administered, wherein the kit comprises:

a) a first boosting composition comprising a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming composition; and

b) a second boosting composition comprising a a source of one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the priming composition; and

instructions for administering the first composition, and then administering the second composition of at a dose that is higher than the dose of the first composition.

13. A kit according to claim 11 or claim 12, comprising two doses of each composition.

14. A kit according to any of claims 11 to 13, wherein the instructions are for administering the second boosting composition at a dose that is about four times greater than the dose of the first boosting composition.

15. Use, a composition or a kit according to any of claims 4 to 14, wherein the first boosting composition and the second boosting composition are the same.

16. Use, a composition or a kit according to any of claims 4 to 14, wherein the first boosting composition and the second boosting composition are different.

17. Use, a composition or a kit according to any of the preceding claims, wherein the priming composition is a DNA plasmid.

18. Use, a composition or a kit according to any of the preceding claims, wherein the boosting composition is a non-replicating or replication-impaired poxvirus.

19. Use, a composition or a kit according to claim 18, wherein the poxvirus is a Modified Vaccinia Virus Ankara.

20. Use, a composition or a kit according to claim 18 or claim 19, wherein the priming composition is a viral vector derived from a virus other than a non-replicating or replication-impaired poxvirus.

21. Use of a recombinant replication-deficient or replication-impaired poxvirus comprising a heterologous polynucleotide encoding one or more epitopes of an antigen in the manufacture of a medicament for inducing an immune response against

the antigen in an individual, wherein the medicament is for sequential administration at a first dose and at a second dose, wherein the second dose is higher than the first dose.

22. Use of a first composition and a second composition in the manufacture of a medicament for sequential administration to an individual to induce an immune response against an antigen, wherein

the first composition comprises a recombinant replication-deficient or replication impaired poxvirus comprising a heterologous polynucleotide encoding one or more epitopes of the antigen; and

the second composition comprises a recombinant replication-deficient or replication impaired poxvirus comprising a heterologous polynucleotide encoding one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the first composition; and wherein

the second composition is for administration at a dose than is higher than the dose of the first composition.

23. A kit for inducing an immune response against an antigen in an individual, wherein the kit comprises:

a) a first composition comprising a recombinant replication-deficient or replication impaired poxvirus comprising a heterologous polynucleotide encoding one or more epitopes of the antigen; and

b) a second composition comprising a recombinant replication-deficient or replication impaired poxvirus comprising a heterologous polynucleotide encoding one or more epitopes of the antigen, including at least one epitope which is the same as an epitope of the first composition; and

instructions for administering a dose of the first composition and then administering a dose of the second composition, wherein the dose of the second composition is higher than the dose of the first composition.

24. A kit according to claim 23 comprising two doses of each of the first composition and the second composition.

25. A kit according to claim 22 or claim 23, wherein the instructions are for administering the second composition at a dose that is about four times greater than the dose of the first composition.

26. Use according to claim 22 or a kit according to any of claims 23 to 25, wherein the first composition and the second composition are the same.

27. Use according to claim 22 or a kit according to any of claims 23 to 25, wherein the first composition and the second composition are different.

28. Use according to claim 21 or 22, or a kit according to any of claims 23 to 27, wherein the poxvirus is a Modified Vaccinia Virus Ankara (MVA).

29. Use, a composition or a kit according to any of the preceding claims, wherein the antigen is a tumour antigen.

30. Use, a composition or a kit according to claim 29, wherein the antigen is a melanoma tumour antigen.

31. Use, a composition or a kit according to any of the preceding claims, wherein the immune response is a CD8+ T cell immune response.

32. Use, a composition or a kit according to any of the preceding claims, wherein the individual is a mammal.

33. Use, a composition or a kit according to claim 32, wherein the mammal is a human.

34. Use of a priming composition and a boosting composition in the manufacture of a medicament for sequential administration to a mammal to treat melanoma; wherein

the priming composition comprises a source of one or more epitopes of melanoma; and

the boosting composition comprises a source of one or more epitopes of melanoma, including at least one epitope which is the same as an epitope of the priming composition, wherein the source of melanoma epitopes is a non-replicating or replication impaired recombinant poxvirus; and wherein

the boosting composition is for sequential administration as a first boosting composition and a second boosting composition, wherein the second boosting composition is for administration at a dose that is higher than the dose of the first boosting composition;

with the proviso that if the source of epitopes in the priming composition is a viral vector, the viral vector in the boosting composition is derived from a different virus.

35. Use according to claim 34, wherein the priming composition is a DNA plasmid.

36. Use according to claim 35, wherein the DNA plasmid is pSG.Mel3

37. Use according to any of claims 34 to 36, wherein the poxvirus is a modified vaccinia virus ankara (MVA).

38. Use according to claim 37, wherein the MVA is MVA.Mel3.

39. Use according to any of claims 34 to 38, wherein the immune response is a CD8+ T cell immune response.

40. Use according to any of claims 34 to 39 wherein the melanoma is a stage III or stage IV melanoma.

41. Use according to any of claims 34 to 40, wherein tumour regression occurs after the second boosting composition is administered.

42. Use according to any of claims 34 to 41, wherein the mammal is a human.

43. Use according to any of claims 35 to 42, wherein the priming composition is for administration at a dose of 2 mg.

44. Use according to any of claims 34 to 43, wherein the first boosting composition is for administration at a dose of from about 5×10^7 pfu to about 1×10^9 pfu.

45. Use according to claim 44, wherein the first boosting composition is for administration at a dose of about 5×10^7 pfu, about 2×10^8 pfu, about 5×10^8 pfu or about 1×10^9 pfu.

46. Use according to any of claims 34 to 45, wherein the second boosting composition is for administration at a dose that is about four times greater than the dose of the first boosting composition.

47. Use according to claim 46, wherein the first boosting composition is for administration at a dose of about 5×10^7 pfu and the second boosting composition is for administration at a dose of about 2×10^8 pfu.

48. Use according to any of claims 34 to 47, wherein the priming composition and each boosting composition are for administration in two doses.

49. A kit for treating melanoma in a mammal, which comprises

a) a priming composition comprising a source of one or more epitopes of melanoma;

b) a boosting composition comprising a source of one or more epitopes of melanoma, including at least one epitope which is the same as an epitope of the priming composition, wherein the source of melanoma epitopes is a non-replicating or replication impaired recombinant poxvirus; and

instructions for administering the boosting composition of b) at a specified dose, followed by administering the boosting composition of b) again at a higher dose than the specified dose;

with the proviso that if the source of epitopes in a) is a viral vector, the viral vector in b) is derived from a different virus.

50. A kit according to claim 49, wherein the priming composition is a DNA plasmid.

51. A kit according to claim 50, wherein the DNA plasmid is pSG.Mel3

52. A kit according to any of claims 49 to 51, wherein the poxvirus is a Modified Vaccinia Virus Ankara (MVA).

53. A kit according to claim 52, wherein the MVA is MVA.Mel3.

54. A kit according to any of claims 49 to 53, wherein the immune response is a CD8+ T cell immune response.

55. A kit according to any of claims 49 to 54 wherein the melanoma is a stage III or stage IV melanoma.

56. A kit according to any of claims 49 to 55, wherein the mammal is a human.

57. A kit according to any of claims 49 to 56, wherein tumour regression occurs after the second boosting composition is administered.

58. A kit according to any of claims 50 to 57, wherein the dose of the priming composition is 2 mg.

59. A kit according to any of claims 49 to 585, wherein the specified dose of the boosting composition is from about 5×10^7 pfu to about 1×10^9 pfu.

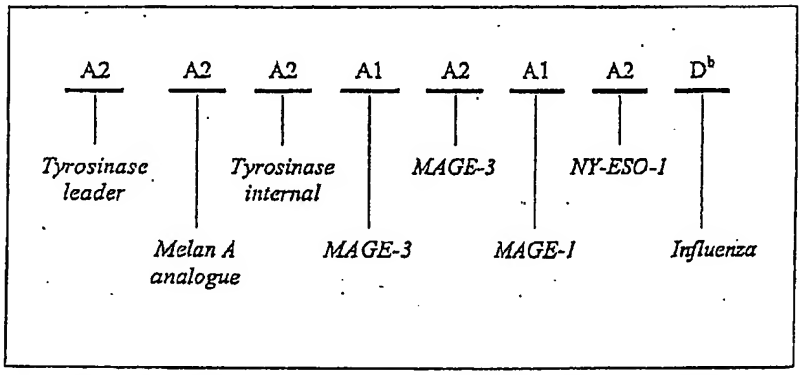
60. A kit according to claim 59, wherein the specified dose of the boosting composition is about 5×10^7 pfu, about 2×10^8 pfu, about 5×10^8 pfu or about 1×10^9 pfu.

61. A kit according to any of claims 49 to 60, wherein the higher dose is four times greater than the specified dose.

62. A kit according to claim 61, wherein the specified dose is about 5×10^7 pfu and the higher dose is about 2×10^8 pfu.

63. A kit according to any of claims 49 to 62, wherein the instructions are for administering the priming composition and each boosting composition are in two doses.

Schematic diagram of the melanoma CTL poly-epitope gene.



CTL epitopes are denoted by solid lines. The HLA molecules associated with each epitope are shown above the solid lines (A1, A2 for human; D^b for murine).

FIGURE 1

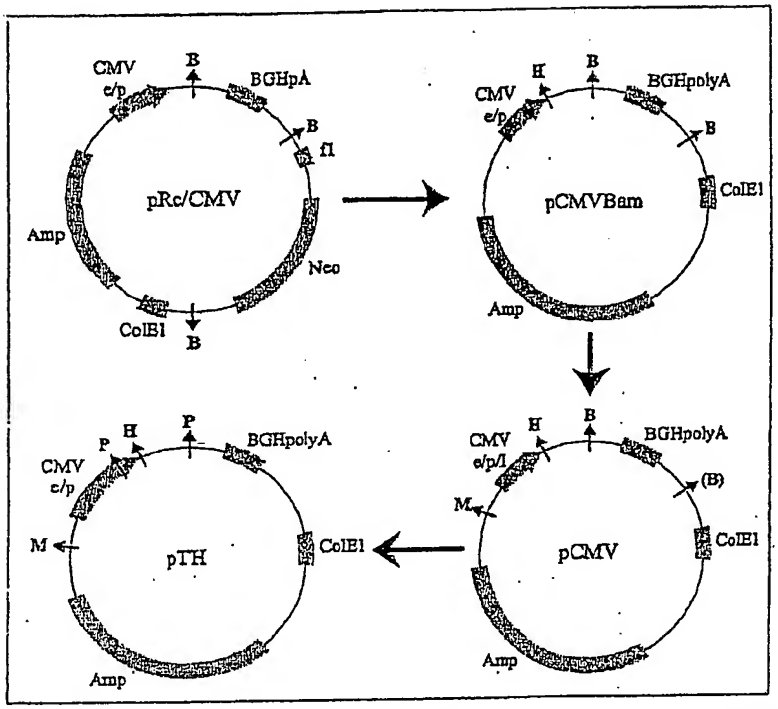
Amino acid sequences of the melanoma and influenza CTL epitopes encoded by the melanoma CTL poly-epitope gene.

Antigen	Location	Peptide sequence	HLA
Tyrosinase leader	1-9	MLLAVLYCL	A2
Melan A analogue	26-35	ELAGIGILTV*	A2
Tyrosinase internal	369-377	YMDGTMSQV**	A2
MAGE-3	168-176	EVDPIGHLY	A1
MAGE-3	271-279	FLWGPRALV	A2
MAGE-1	161-169	EADPTGHSY	A1
NY-ESO-1	155-167	SLLMWITQCFL	A2
Influenza nuclear protein	366-374	ASNENMDAM	D^b

* wild type sequence EAAGIGILTV
** wild type sequence YMNGTMSQV

FIGURE 2

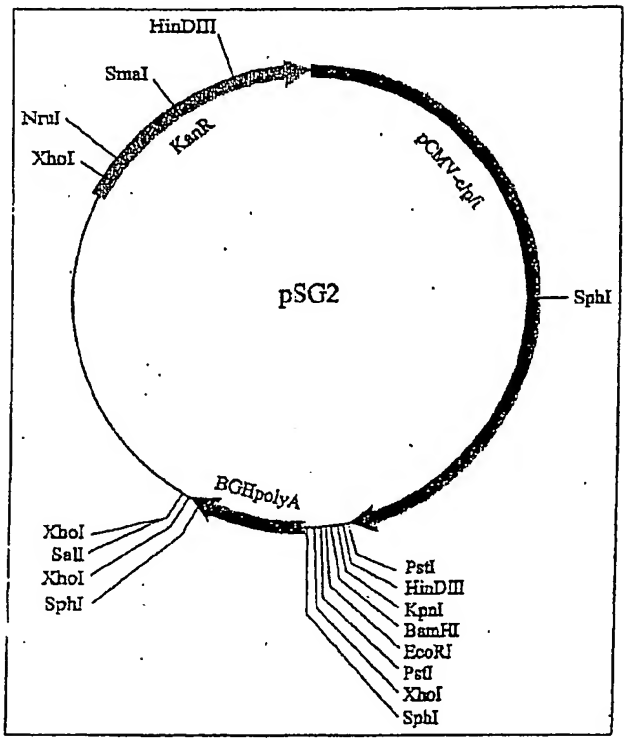
Construction of plasmid pTH.



CMVe/p – hCMV enhancer/promoter region; CMVe/p/I – hCMV enhancer/promoter/intron region; BGHpolyA – bovine growth hormone polyadenylation signal; Amp - β-lactamase gene; ColE1 – *E.coli* origin of replication; Neo – neomycin resistance gene; f1 – M13 origin of replication; B – *BamH*I; H – *HinD*III; M – *Mlu*I; P – *Pst*I.

FIGURE 3

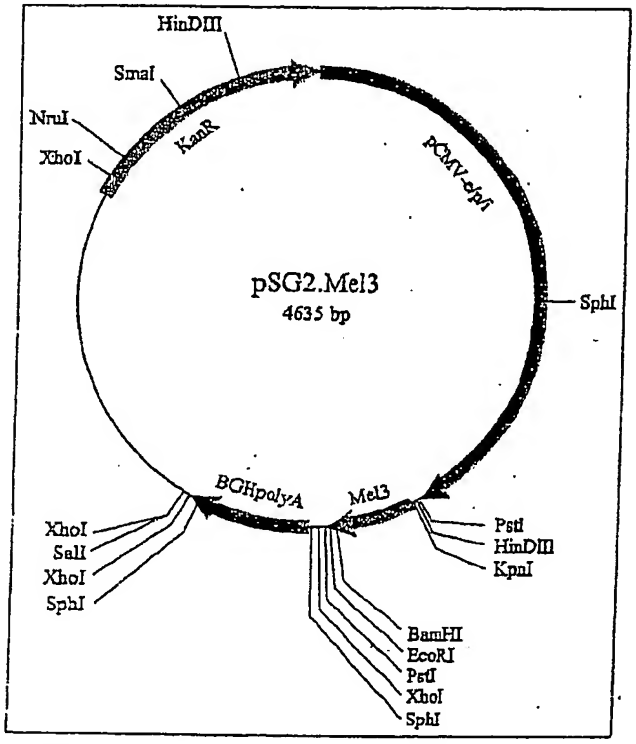
Map of plasmid pSG2.



CMV-e/p/i – hCMV enhancer/promoter/intron A region; BGHpolyA – bovine growth hormone polyadenylation signal; KanR – kanamycin resistance gene.

FIGURE 4

Map of plasmid pSG2.Mel3.



CMV-e/p/i – hCMV enhancer/promoter/intron A region; BGHpolyA – bovine growth hormone polyadenylation signal; Mel3 – melanoma CTL poly-epitope gene; KanR – kanamycin resistance gene.

FIGURE 5

Nucleotide sequence of the assembled CTL poly-epitope gene cassette and predicted amino acid sequence.

AGATCTGCCGCCACCATGTTACTAGCTGTTTTGTACTGCCTGGAACTAGCAGGGATC
M L L A V L Y C L E L A G I
Tyrosinase leader *Melan A*

GGCATATTGACAGTGTATATGGATGGAACAATGTCCCAGGTAGGATCTGAAGTCGAT
G I L T V Y M D G T M S Q V G S E V D
analogue *Tyrosinase internal*

CCAATCGGACATTTGTACTTCCTGTGGGGTCCAAGAGCCCTCGTTGAAGCAGACCCC
P I G H L Y F L W G P R A L V E A D P
MAGE-3 *MAGE-3*

ACCGGACACTCCTATGGATCTCAGCTTTCCCTGTTGATGTGGATCACGCAGTGCTTT
T G H S Y G S Q L S L L M W I T Q C F
MAGE-1 *NY-ESO-1*

CTGGCTTCAAATGAAAACATGGATGCTATGTGAGGATCC
L A S N E N M D A M *
Influenza NP

FIGURE 6

Complete nucleotide sequence of plasmid pSG2.Mel3.

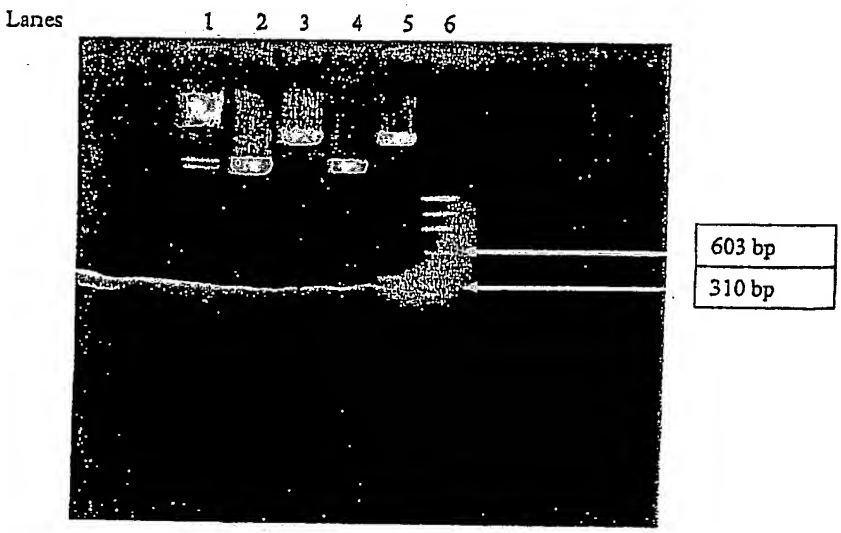
TGTGAGTTTCTGTGTAACTGATATCGCCATTTTTCCAAAAGTGATTTTTGGGCATACGCGAT
ATCTGGCGATAGCGCTTATATCGTTTACGGGGGATGGCGATAGACGACTTTGGTGACTTGGG
CGATTCTGTGTGTCGCAAATATCGCAGTTTCGATATAGGTGACAGACGATATGAGGCTATAT
CGCCGATAGAGGCGACATCAAGCTGGCACATGGCCAATGCATATCGATCTATACATTGAATC
AATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAATATTGGCTATTGG
CCATTGCATACGTTGTATCCATATCATAATATGTACATTTATATTGGCTCATGTCCAACATT
ACCGCCATGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAG
TTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGA
CCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAAT
AGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTAC
ATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCC
TGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATT
AGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGT
TTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCAC
CAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGG
TAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCT
GGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCGATCCAGCCTCCGC
GGCCGGGAACGGTGCATTGGAACGCGGATTCCCCGTGCCAAGAGTGACGTAAGTACCGCCTA
TAGAGTCTATAGGCCCACCCCCTTGGCTTCTTATGCATGCTATACTGTTTTTGGCTTGGGGT
CTATACACCCCCGCTTCCTCATGTTATAGGTGATGGTATAGCTTAGCCTATAGGTGTGGGTT
ATTGACCATTATTGACCACTCCCCTATTGGTGACGATACTTTCCATTACTAATCCATAACAT
GGCTCTTTGCCACAACTCTCTTTATTGGCTATATGCCAATACACTGTCCTTCAGAGACTGAC
ACGGACTCTGTATTTTTACAGGATGGGGTCTCATTTATTATTTACAAATTCACATATACAAC
ACCACCGTCCCCAGTGCCCGCAGTTTTTATTAAACATAACGTGGGATCTCCACGCGAATCTC
GGGTACGTGTTCCGGACATGGGCTCTTCTCCGGTAGCGGCGGAGCTTCTACATCCGAGCCCT
GCTCCCATGCCTCCAGCGACTCATGGTCGCTCGGCAGCTCCTTGCTCCTAACAGTGGAGGCC
AGACTTAGGCACAGCACGATGCCCACCACCACCAGTGTGCCGCACAAGGCCGTGGCGGTAGG
GTATGTGTCTGAAAATGAGCTCGGGGAGCGGGCTTGCACCGCTGACGCATTTGGAAGACTTA
AGGCAGCGGCAGAAGAAGATGCAGGCAGCTGAGTTGTTGTGTTCTGATAAGAGTCAGAGGTA
ACTCCCGTTGCGGTGCTGTTAACGGTGGAGGGCAGTGTAGTCTGAGCAGTACTCGTTGCTGC
CGCGCGCGCCACCAGACATAATAGCTGACAGACTAACAGACTGTTCCTTTCCATGGGTCTTT
TCTGCAGTCACCGTCCTTGACACGAAGCTTGGTACCGAGCTCGGATCTGCCGCCACCATGGT
ACTAGCTGTTTTGTACTGCCTGGAACTAGCAGGGATCGGCATATTGACAGTGTATATGGATG
GAACAATGTCCCAGGTAGGATCTGAAGTCGATCCAATCGGACATTTGTACTTCCTGTGGGGT
CCAAGAGCCCTCGTTGAAGCAGACCCCACCGGACACTCCTATGGATCTCAGCTTTCCCTGTT
GATGTGGATCACGCAGTGCTTTCTGGCTTCAAATGAAAACATGGATGCTATGTGAGGATCCA
CTAGTAACGGCCGCCAGTGTGCTGGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGA
GCATGCATCTAGAGGGCCCTATTCTATAGTGTCACCTAAATGCTAGAGCTCGCTGATCAGCC
TCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGAC
CCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTC
TGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGG
GAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAAC
CAGCTGGGGCTCGAGGGGGGATCGATCCGTCGACCTCGAGAGCTTGGCGTAATCATGGTCAT
AGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGC
ATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTC
ACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCG
CGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGC

FIGURE 7A

TCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCAC
AGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACC
GTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAA
AATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCC
CCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCG
CCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCG
GTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTG
CGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGG
CAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTG
AAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAA
GCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTA
GCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGAT
CCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTT
GGTCATGAACAATAAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTGTTATGAGCCAT
ATTCAACGGGAAACGTCTTGCTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTATA
TGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGATTGTATG
GGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTT
ACAGATGAGATGGTCAGACTAAACTGGCTGACGGAATTTATGCCTCTTCCGACCATCAAGCA
TTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTGCGATCCCCGGGAAAACAGCAT
TCCAGGTATTAGAAGAATATCCTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTC
CTGCGCCGGTTGCATTCGATTCCTGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTTCG
TCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTGATGACG
AGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAGCTTTTGCCATTCTCA
CCGGATTCAGTCGTCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAA
ATTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCA
TCCTATGGAACTGCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATAT
GGTATTGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTA
ATCAGAATTGGTTAATTGGTTGTAACACTGGCAGAGCATTACGCTGA

FIGURE 7B

Restriction enzyme analysis of plasmids pSG2 and pSG2.Mel3.



Lanes:

1 λ*HinD*III marker
2 pSG2 *BamHI*/*HinD*III
3 pSG2 *PstI*
4 pSG2.Mel3 *BamHI*/*HinD*III
5 pSG2.Mel3 *PstI*
6 ϕ*Xho*I marker

FIGURE 8

Oligonucleotides used to construct the melanoma CTL poly-epitope gene.

A1	GATCTGCCGCCACCATGTTACTAGCTGTTTTGTACTGCCTGGAACTAGCAGGGATC
A2	GGCATATTGACAGTGTATATGGATGGAACAATGTCCCAGGTAG
A3	CTAGTTCCAGGCAGTACAAAACAGCTAGTAACATGGTGGCGGCA
A4	GATCCTACCTGGGACATTGTTCCATCCATATACACTGTCAATATGCCGATCCCTG
B1	GATCTGAAGTCGATCCAATCGGACATTTGTACTTCCTGTGGGGT
B2	CCAAGAGCCCTCGTTGAAGCAGACCCCACCGGACACTCCTATG
B3	GGAAGTACAAATGTCCGATTGGATCGACTTCA
B4	GATCCATAGGAGTGTCCGGTGGGGTCTGCTTCAACGAGGGCTCTTGGACCCCACA
C1	GATCTCAGCTTTCCCTGTTGATGTGGATCACGCAGTGCTTTCTG
C2	GCTTCAAATGAAAACATGGATGCTATGTGAG
C3	ACTGCGTGATCCACATCAACAGGGAAAGCTGA
C4	GATCCTCACATAGCATCCATGTTTTCATTTGAAGCCAGAAAGC

FIGURE 9

Oligonucleotide primers used for the sequencing the poly-epitope gene in plasmid pSG2.Mel3

THP1	GCCACCAGACATAATAGCTG
THP2	ACAGATGGCTGGCAACTAGA

FIGURE 10

Oligonucleotide primers used for sequencing of the melanoma poly-epitope gene in MVA.Mel3

Forward primer

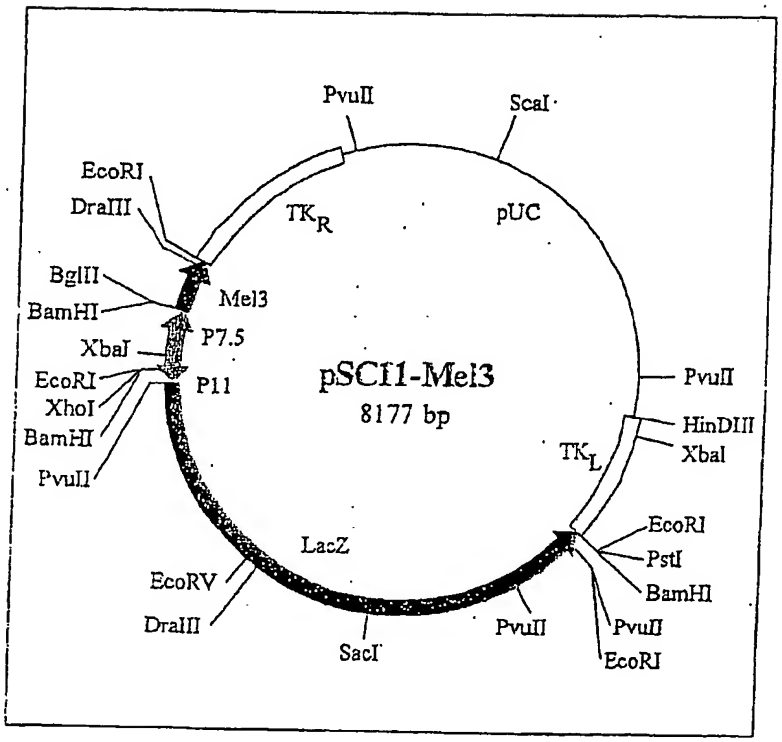
5' ACTCAGATCTCCACCATGTTACTAGCTGTT 3'

Reverse primer

5' ACGAATTCTCACATAGCATCCATGTTTTCA 3'

FIGURE 11

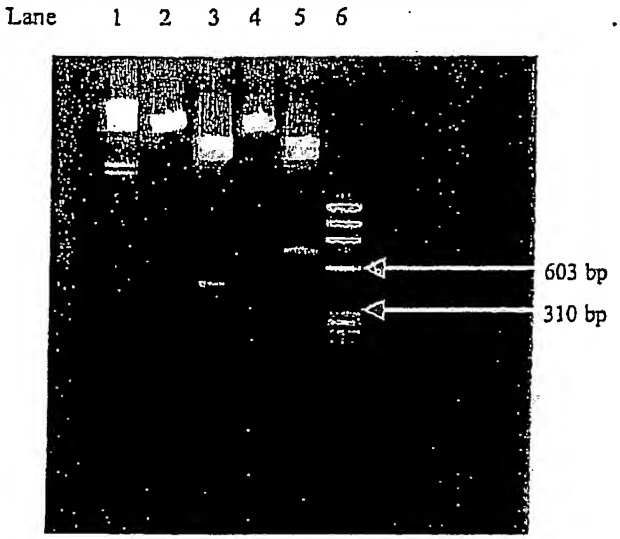
Map of plasmid pSC11.Mel3



TK$_L$ – thymidine kinase left fragment; TK$_R$ – thymidine kinase right fragment; P11 – vaccinia P11 promoter; P7.5 – vaccinia P7.5 promoter; Mel3 – melanoma CTL poly-epitope gene.

FIGURE 12

Restriction enzyme analysis of plasmids pSC11.Mel3 and pSC11*



Lanes:

1	λ DNA/*HinD*III marker
2	pSC11* *BglII/NotI*
3	pSC11* *EcoR*1
4	pSC11.Mel3 *BglII/NotI*
5	pSC11.Mel3 *EcoR*1
6	ϕX174 DNA/*Hae*III marker

FIGURE 13

Nucleotide sequence of the melanoma poly-epitope gene in recombinant MVA.Mel3 and predicted amino acid sequence

```
Fwd-mel3          ------------------CAGTCGACGTACAGATCTGCCGCCACCATGTTACTAGCTGTT 50
reverse-mel3      AGAACAGTGACGGATCCCCAGTCGACGTACAGATCTGCCGCCACCATGTTACTAGCTGTT 780
                              **********************************************
Amino acid seq                                                M  L  L  A  V

Fwd-mel3          TTGTACTGCCTGGAACTAGCAGGGATGGCATATTGACAGTGTATATGGATGGAACAATG 110
reverse-mel3      TTGTACTGCCTGGAACTAGCAGGGATGGCATATTGACAGTGTATATGGATGGAACAATG 640
                  ************************************************************
Amino acid seq     L  Y  C  L  E  L  A  G  I  G  I  L  T  V  Y  M  D  G  T  M

Fwd-mel3          TCCCAGGTAGGATCTGAAGTCGATCCAATCGGACATTTGTACTTCCTGTGGGGTCCAAGA 170
reverse-mel3      TCCCAGGTAGGATCTGAAGTCGATCCAATCGGACATTTGTACTTCCTGTGGGGTCCAAGA 900
                  ************************************************************
Amino acid seq     S  Q  V  G  S  E  V  D  P  I  G  H  L  Y  F  L  W  G  P  R

Fwd-mel3          GCCCTCGTTGAAGCAGACCCCACCGGACACTCCTATGGATCTCAGCTTTCCCTGTTGATG 230
reverse-mel3      GCCCTCGTTGAAGCAGACCCCACCGGACACTCCTATGGATCTCAGCTTTCCCTGTTGATG 960
                  ************************************************************
Amino acid seq     A  L  V  E  A  D  P  T  G  H  S  Y  G  S  Q  L  S  L  L  M

Fwd-mel3          TGGATCACGCAGTGCTTTCTGGCTTCAAATGAAAACATGGATGCTATGTGAGGATCTGTA 290
reverse-mel3      TGGATCACGCAGTGCTTTCTGGCTTCAAATGAAAACATGGATGCTATGTGAGGATCTGTA 1020
                  ************************************************************
Amino acid seq     W  I  T  Q  C  F  L  A  S  N  E  N  M  D  A  M  *

Fwd-mel3          CGGGCCCGTACGGTACCGTACGCGGCCGCTGGGAATTCTGTGAGCGTATGGCAAACGAAG 350
reverse-mel3      CGGGCCCGTACGGTACCGTACGCGGCCGCTGGGAATTCTGTGAGNGTA-GGCAAA-GAAG 1078
                  ************************************************ *** ****** ****

Fwd-mel3          GAAAAATAGTTATAGTAGCCGCACTCGATGGGACATTTTAANNNNCCTCCCTCNNTGNIT 410
reverse-mel3      GAAAAA-------AGTGG----------------------ATAGA---C---------- 1095
                  ******       *** *                      *     *

Fwd-mel3          TTTNTNANC 419
reverse-mel3      ---A--AAN 1099
```

FIGURE 14

Patient Baseline Characteristics

	Group 1 (N=8)	Group 2 (N=5)	Group 3 (N=5)	Group 4 (N=7)	Group 5 (N=6)	Total (N=31)
Age						
Mean	58	68	58	61	55	60
Range	27 - 76	47 - 86	42 - 68	41 - 76	38 - 64	27-86
Gender						
Male	4	3	2	1	3	13
Female	4	2	3	6	3	18
Stage						
IV	7	4	5	6	6	28
III	1	1	0	1	0	3
Metastatic sites						
Other*	1	0	1	3	1	6
Liver	0	0	0	2	1	3
Lung	3	2	4	0	4	13
Nodes	3	1	0	1	0	5
Skin	1	2	0	1	0	4
Prior therapy						
Surgery	7	5	5	7	6	30
Radiotherapy	1	0	1	2	4	8
Chemotherapy	2	2	2	4	3	13
Immunotherapy	1	0	2	1	2	6
Laser therapy	2	2	0	0	0	4
Gene therapy	0	1	0	0	0	1

* Bone, kidney, pancreas, chest wall, stomach, adrenal, pancreas, bladder, intra-abdominal

- Age was balanced between groups
- 58% of patients were female
- 90% of patients had stage IV disease.
- Patients in groups 1-3 had disease predominantly limited to skin, nodes and lung.
- Patients in groups 4 and 5 had disease predominantly in lung, liver and other organs.
- Prior chemotherapy and radiotherapy was more prevalent in groups 4 & 5.

FIGURE 15

Safety Summary

SAFETY	Low Dose (groups 1-3) (n=18)	High Dose (groups 4, 5, 7) (n=18)	MVA Alone (Group 6) (n=5)
N(%)			
Grade 3/4	1 (6%)	4 (22%)	2 (40%)
Injection Site	13 (72%)	14 (78%)	5 (100%)
Pyrexia	1 (6%)	8 (44%)	1 (20%)
Rigors	0	6 (33%)	2 (40%)

Figure 16

Treatment Related AE by Dose

Group		1 (n=8)	2 (n=5)	3 (n=5)	4 (n=7)	5 (n=6)	6 (n=5)	7 (n=5)	Total (N=41)	
DNA 1	N									36
	Pts	3	3	2	2	1	n/a	2	13	36%
	(Evs)	(3)	(4)	(2)	(5)	(1)		(4)	(21)	(9%)
	[Mean]	[1]	[1.3]	[1]	[2.5]	[1]		[2]	[1.6]	
DNA 2	N									26
	Pts	4	n/a	2	2	1	n/a	n/a	9	35%
	(Evs)	(8)		(5)	(2)	(1)			(16)	(7%)
	[Mean]	[2]		[2.5]	[1]	[1]			[1.8]	
MVA 1	N									40
	Pts	4	4	4	5	6	5	5	33	82%
	(Evs)	(9)	(11)	(9)	(16)	(23)	(11)	(13)	(92)	(40%)
	[Mean]	[2.3]	[2.8]	[2.3]	[3.2]	[3.8]	[2.2]	[2.6]	[2.8]	
MVA 2	N									37
	Pts	5	2	2	4	5	5	4	27	73%
	(Evs)	(11)	(7)	(10)	(8)	(10)	(7)	(10)	(63)	(28%)
	[Mean]	[2.2]	[3.5]	[5]	[2]	[2]	[1.4]	[2.5]	[2.3]	

Figure 17A

Dose Limiting Toxicity

- 15 vaccine related AE NCI-CTC Grade 3/4 reported by 9 patients
- All Grade 3/4 were post MVA and mostly local injection site reactions
- No patients were withdrawn due to grade 3/4 Toxicity

Patient	Group	Event	Grade	SAE
001	1	Fatigue (Post MVA 1)	3	
		Fatigue (Post MVA 2)	3	
011	3	Injection site reaction	3	
046	4	Injection site reaction	3	
021	5	Bone pain	3	
022	5	Injection site reaction	3	
		Injection site reaction	3	
023	5	Injection site reaction	3	
026	6	Petechiae	3	
026		Tachycardia	3	Yes
026		Anaemia	3	Yes
027	6	Injection site reaction	3	
074	7	Injection site reaction	3	
074		Injection site reaction	3	
074		Syncope	4	Yes

Figure 17B

Injection Site Reactions (Day 0 & Day 7 Post Injection)

Group		1 (n=8)	2 (n=5)	3 (n=5)	4 (n=7)	5 (n=6)	6 (n=5)	7 (n=5)	Total (N=41)	
DNA 1	N									36
	Mild	0	1	0	2	2	n/a	0	5	(14%)
	Moderate	0	0	0	0	0		0	0	
	Severe	0	0	0	0	0		0	0	
DNA 2	N									26
	Mild	0	n/a	0	0	0	n/a	0	0	(0%)
	Moderate	0		0	0	0		0	0	
	Severe	0		0	0	0		0	0	
MVA 1	N									40
	Mild	3	3	3	1	2	3	1	16	(40%)
	Moderate	2	2	1	4	2	1	3	15	(37%)
	Severe	0	0	1	1	2	1	1	6	(15%)
MVA 2	N									37
	Mild	4	2	3	1	4	4	3	21	(57%)
	Moderate	0	0	1	3	0	1	1	6	(16%)
	Severe	0	0	0	1	1	0	1	3	(8%)

Figure 18

Summary of responders by group

Group (Number)	1 (N=8)	2 (N=5)	3 (N=5)	4 (N=7)	5 (N=6)	7 (N=5)	6 (N=5)	Overall (N=41)
Immunologically Evaluable	5	5	5	5	6	5	5	36
Melan-A Tetramer	3	2	3	4	5	4	2	23 (66%)
Melan-A ELISPOT	2	1	1	2	1	2	1	11 (31%)

Figure 19

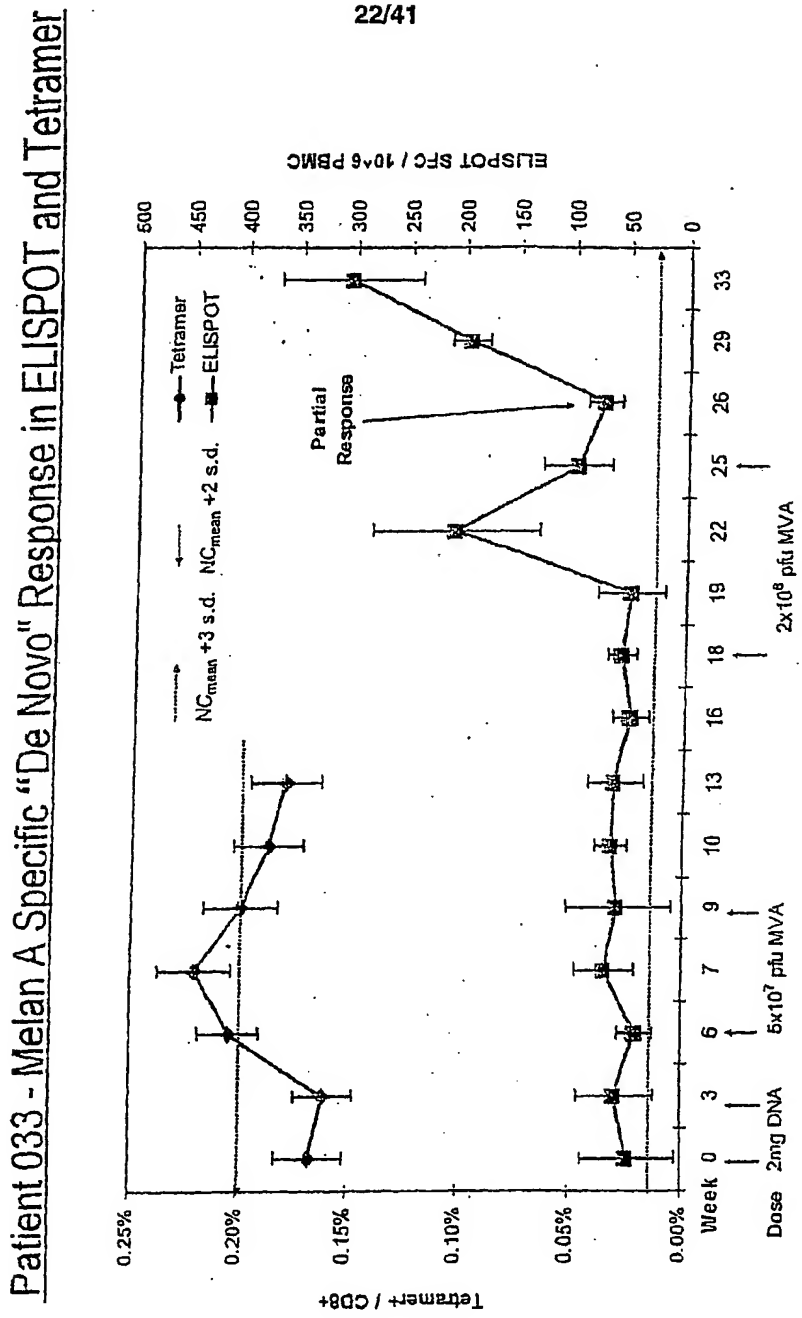

Patient 033 - Melan A Specific "De Novo" Response in ELISPOT and Tetramer
Tetramer+ / CD8+
ELISPOT SFC / 10^6 PBMC
Tetramer
ELISPOT
NC_mean +3 s.d.
NC_mean +2 s.d.
Partial Response
Week 0 3 6 7 9 10 13 16 18 19 22 25 26 29 33
Dose 2mg DNA 5x10^7 pfu MVA 2x10^8 pfu MVA

FIGURE 20

Figure 21: Patient 047 - Melan A Specific "Boosted" Response (ELISPOT and Tetramer)

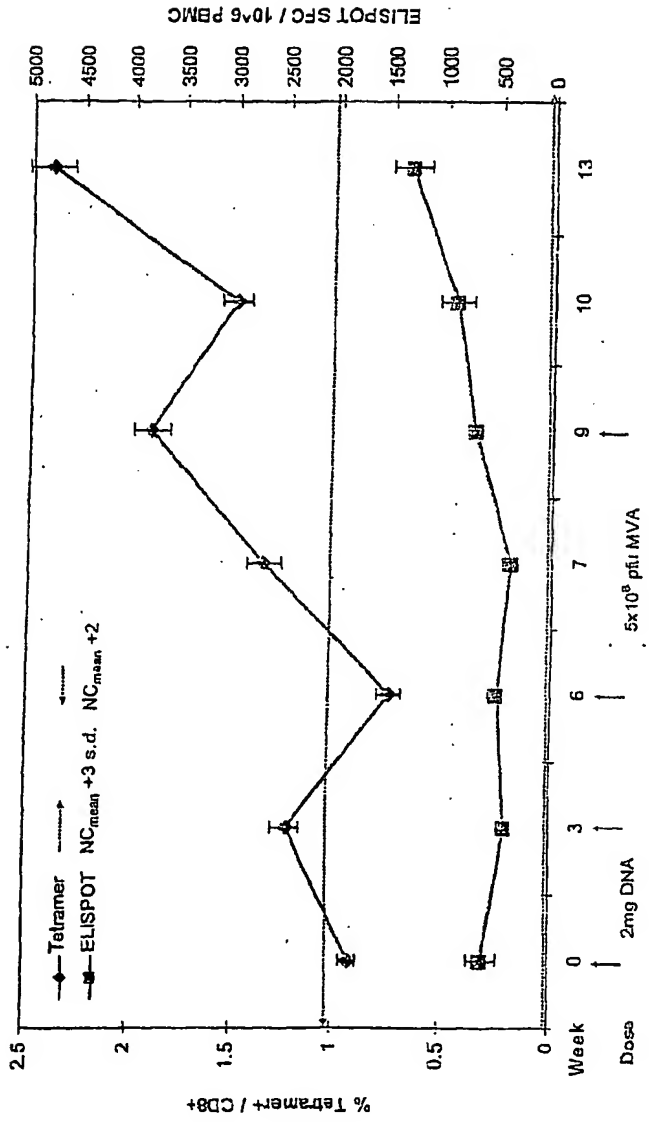


FIGURE 21

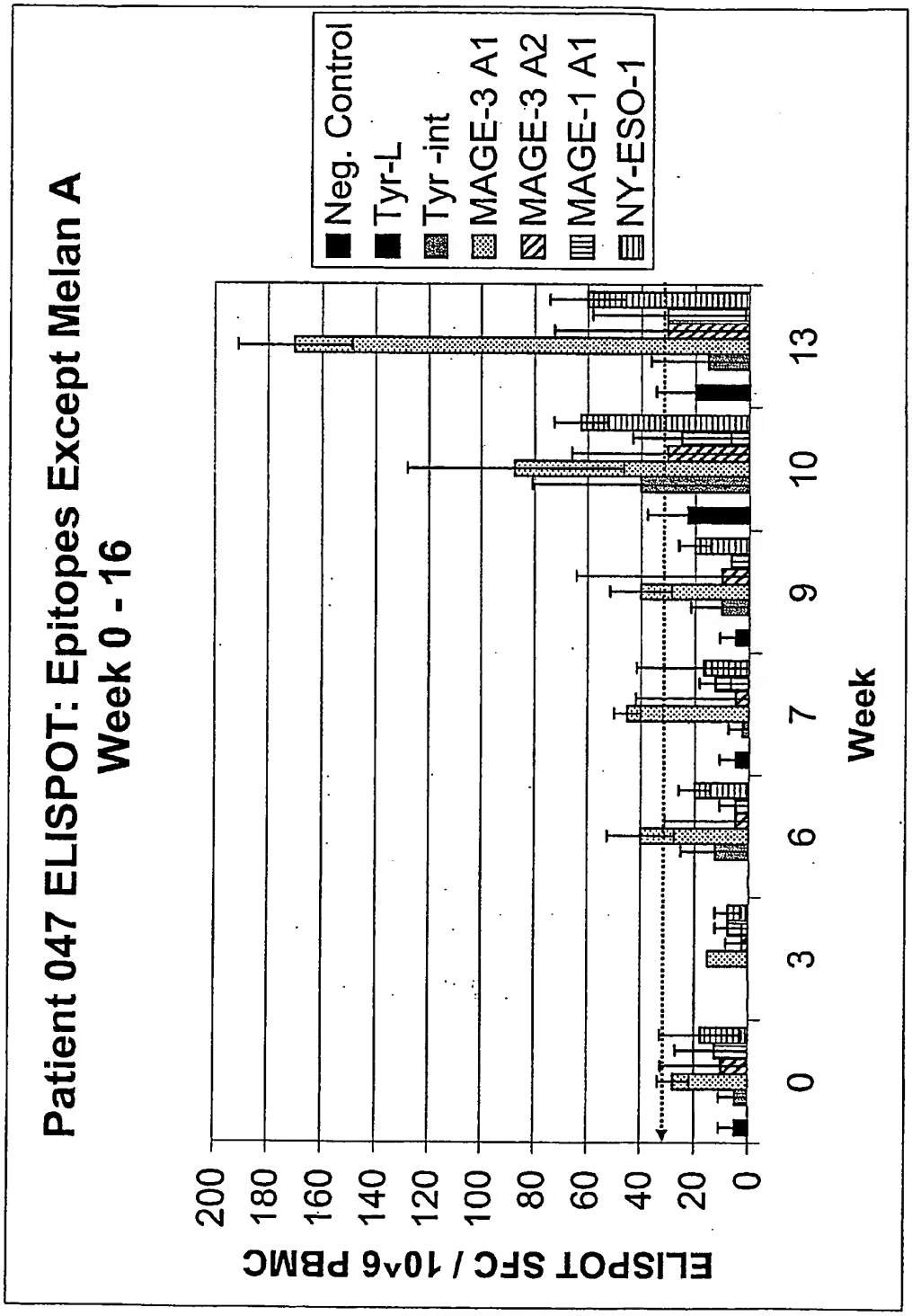

Patient 047 ELISPOT: Epitopes Except Melan A
Week 0 - 16
Neg. Control
Tyr-L
Tyr -int
MAGE-3 A1
MAGE-3 A2
MAGE-1 A1
NY-ESO-1
ELISPOT SFC / 10^6 PBMC
0
20
40
60
80
100
120
140
160
180
200
0
3
6
7
9
10
13
Week

Figure 22

Best Overall Tumour Responses

Group	1 (n=8)	2 (n=5)	3 (n=5)	4 (n=7)	5 (n=6)	6 (n=5)	7 (n=5)	Total (N=41)
Complete Response (CR)	0	0	0	0	0	0	0	0 CR (0%)
Partial Response (PR)	1	0	0	0	0	0	0	1 PR (2%)
Stable Disease[b] (SD)	2	1[a]	0	1	1	0	1,1[a]	7 SD (12%) MR (5%)
Progressive Disease (PD)	5	5	5	6	5	5	3	33 PD (83%)

a – Mixed Response (PR despite one new lesion, unconfirmed at follow up scan)

b - SD TTP $\geq$ 24 weeks

Figure 23A

- Patient 033
- 64 year old male. Stage III disease involving lymph nodes only

Week	LDSUM (mm)	Regression	Overall Response
Baseline	141	-	-
9	120	-15%	SD
17	105	-26%	SD
26	98	-30%	PR
31	99	-30%	PR
42	<84	-40%	PR
48	<89	-37%	PR
72	<73	-48%	PR

Figure 23B

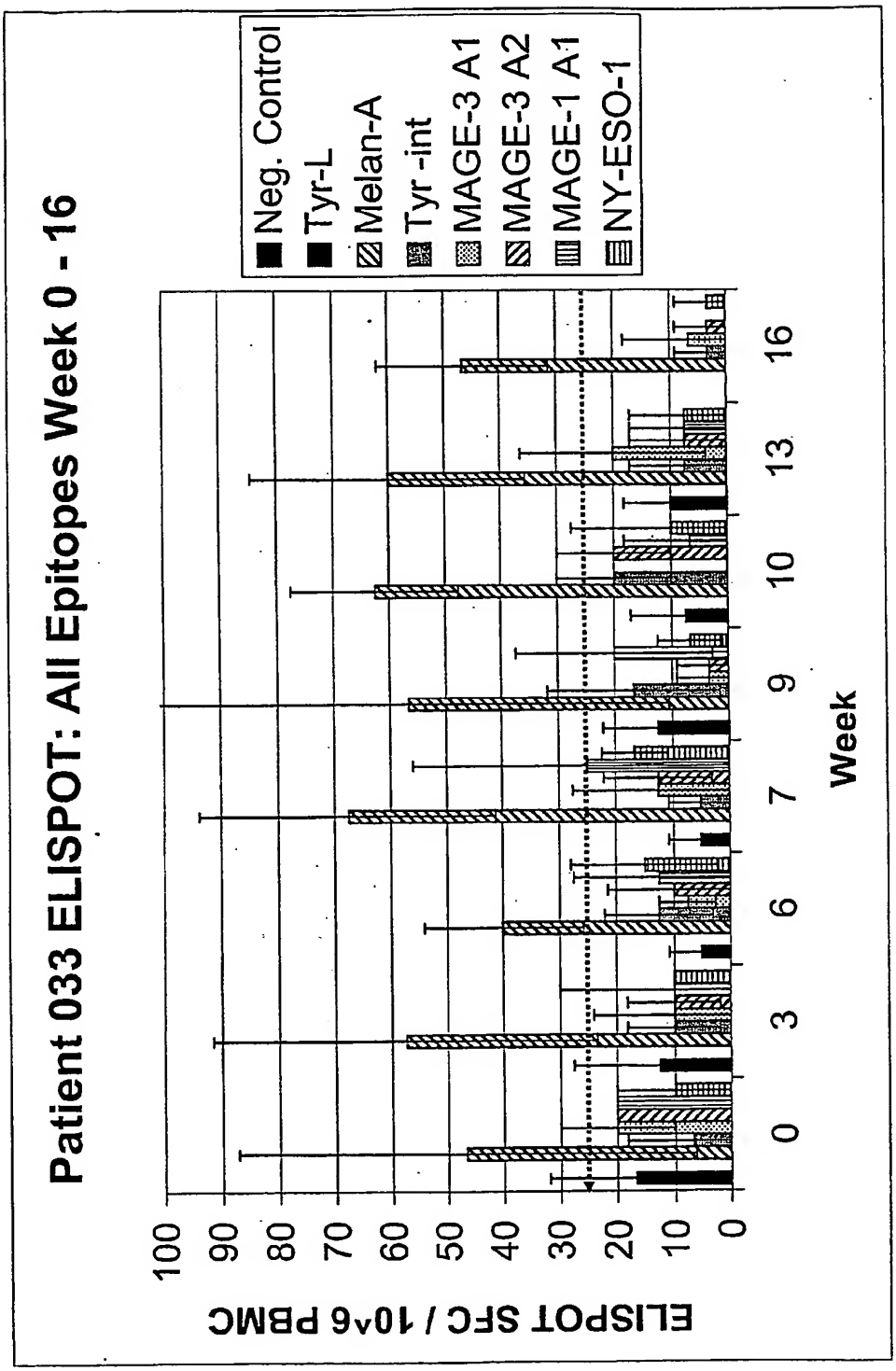

Patient 033 ELISPOT: All Epitopes Week 0 - 16
Neg. Control
Tyr-L
Melan-A
Tyr -int
MAGE-3 A1
MAGE-3 A2
MAGE-1 A1
NY-ESO-1
ELISPOT SFC / 10^6 PBMC
100
90
80
70
60
50
40
30
20
10
0
0
3
6
7
9
10
13
16
Week

Figure 24

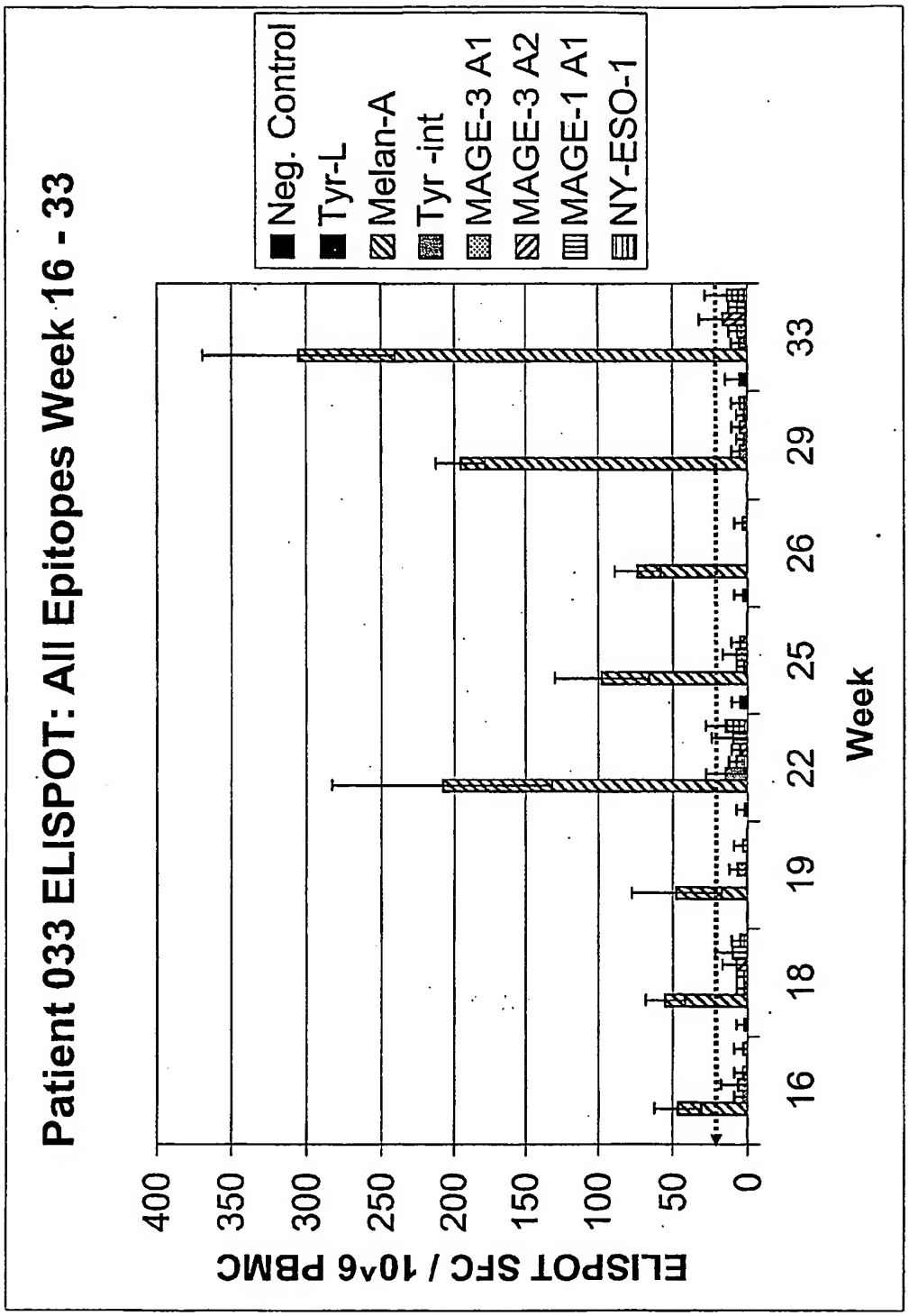

Patient 033 ELISPOT: All Epitopes Week 16 - 33
Neg. Control
Tyr-L
Melan-A
Tyr -int
MAGE-3 A1
MAGE-3 A2
MAGE-1 A1
NY-ESO-1
ELISPOT SFC / 10^6 PBMC
0
50
100
150
200
250
300
350
400
16
18
19
22
25
26
29
33
Week

Figure 25

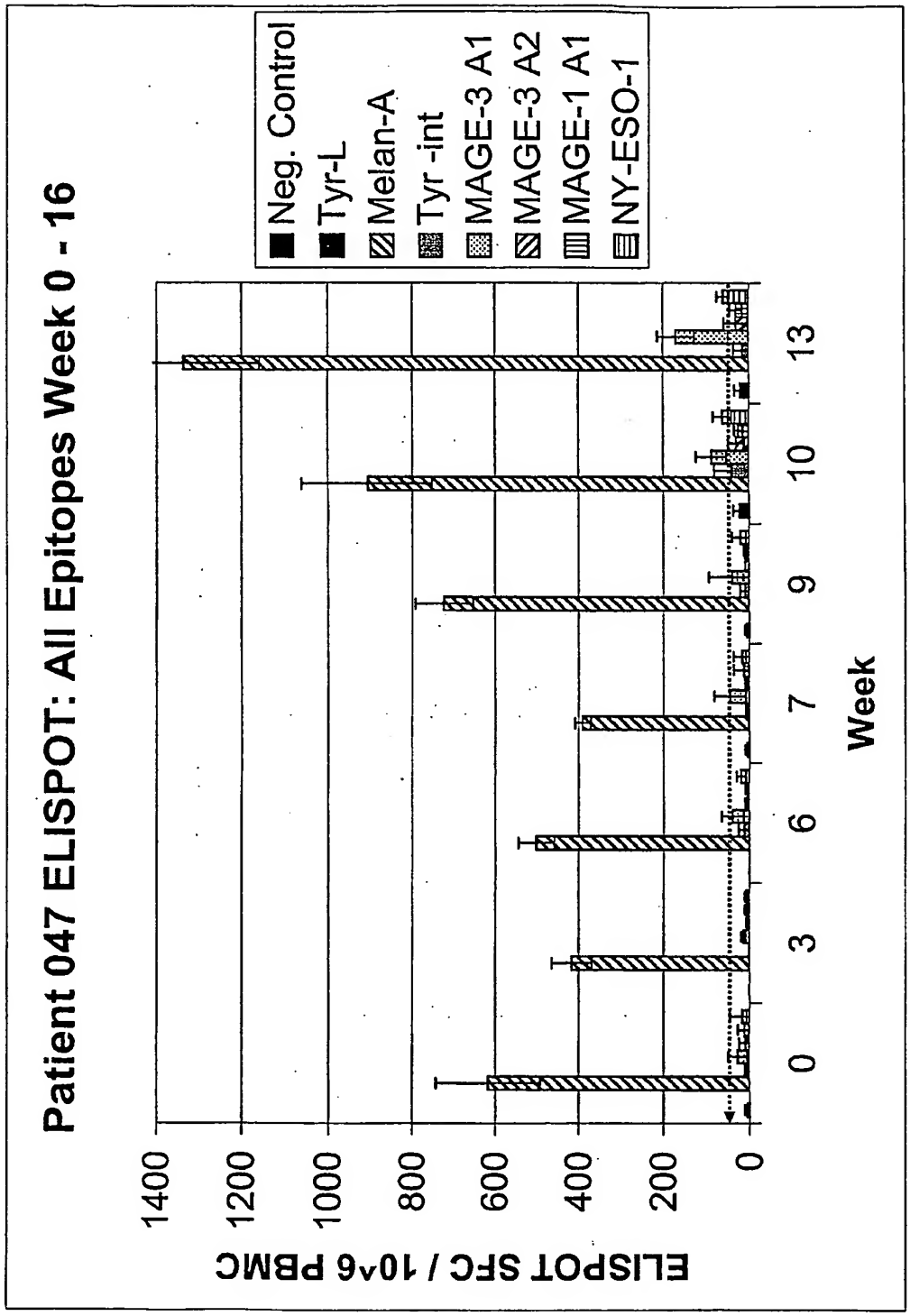

Patient 047 ELISPOT: All Epitopes Week 0 - 16
Neg. Control
Tyr-L
Melan-A
Tyr -int
MAGE-3 A1
MAGE-3 A2
MAGE-1 A1
NY-ESO-1
ELISPOT SFC / 10^6 PBMC
1400
1200
1000
800
600
400
200
0
0
3
6
7
9
10
13
Week

Figure 26

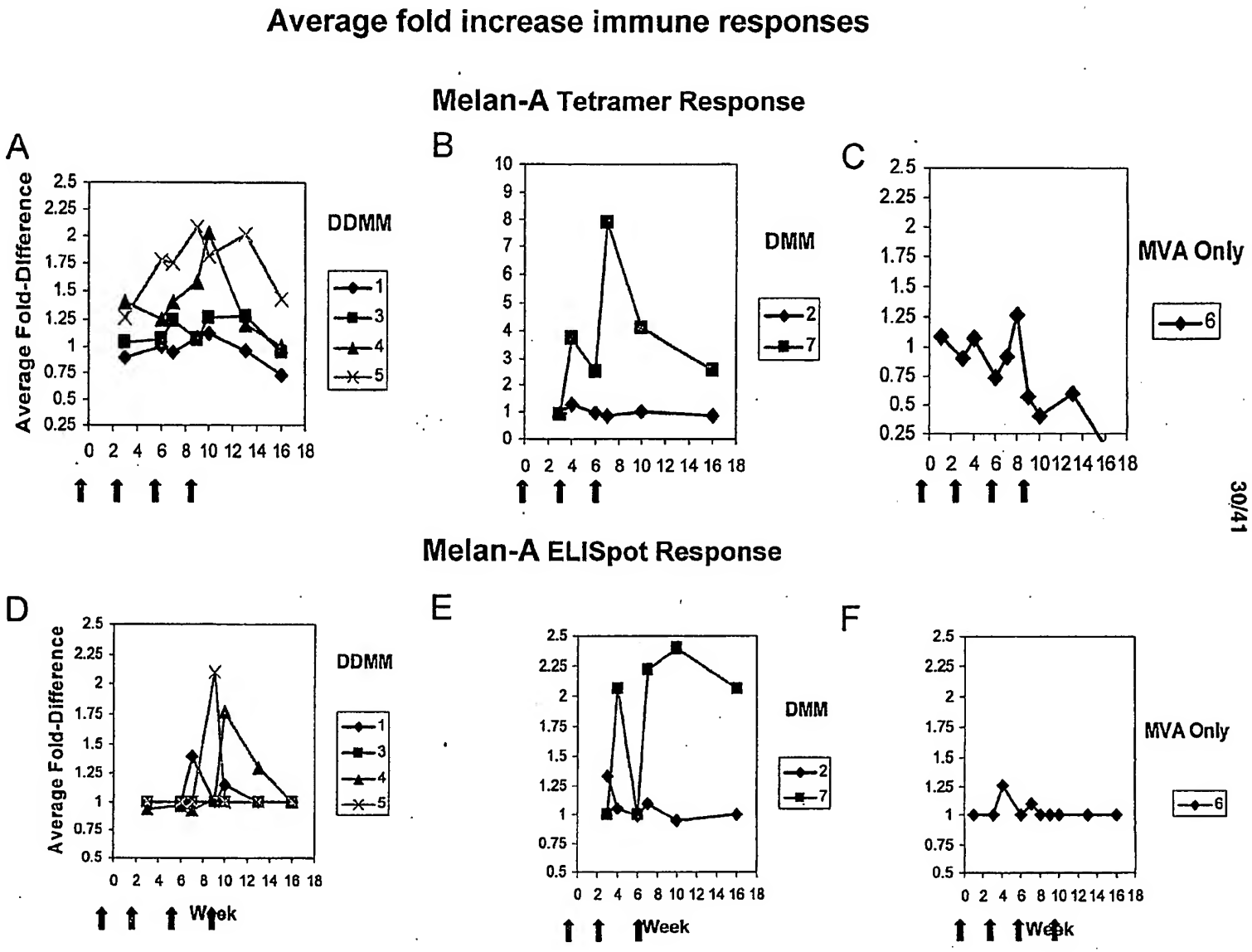

Average fold increase immune responses
Melan-A Tetramer Response
A
Average Fold-Difference
DDMM
1
3
4
5
B
DMM
2
7
C
MVA Only
6
Melan-A ELISpot Response
D
Average Fold-Difference
DDMM
1
3
4
5
Week
E
DMM
2
7
Week
F
MVA Only
6
Week

Figure 27

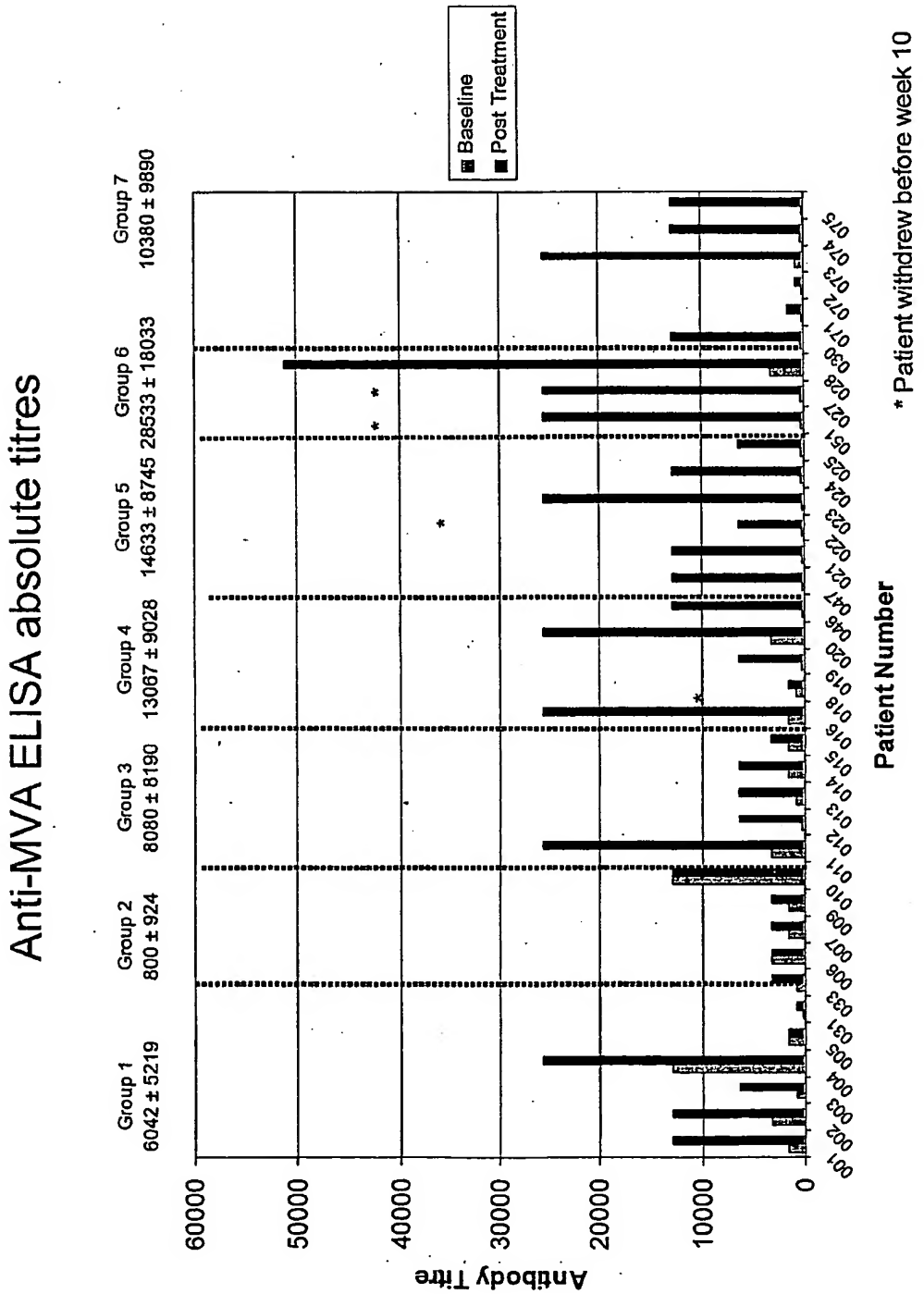

Anti-MVA ELISA absolute titres
Group 1 6042 ± 5219
Group 2 800 ± 924
Group 3 8080 ± 8190
Group 4 13067 ± 9028
Group 5 14633 ± 8745
Group 6 28533 ± 18033
Group 7 10380 ± 9890
Baseline
Post Treatment
Antibody Titre
60000
50000
40000
30000
20000
10000
0
001 002 003 004 005 031 033 006 007 009 010 011 012 013 014 015 016 018 019 020 046 047 021 022 023 024 025 051 027 028 030 071 072 073 074 075
Patient Number
* Patient withdrew before week 10

Figure 28

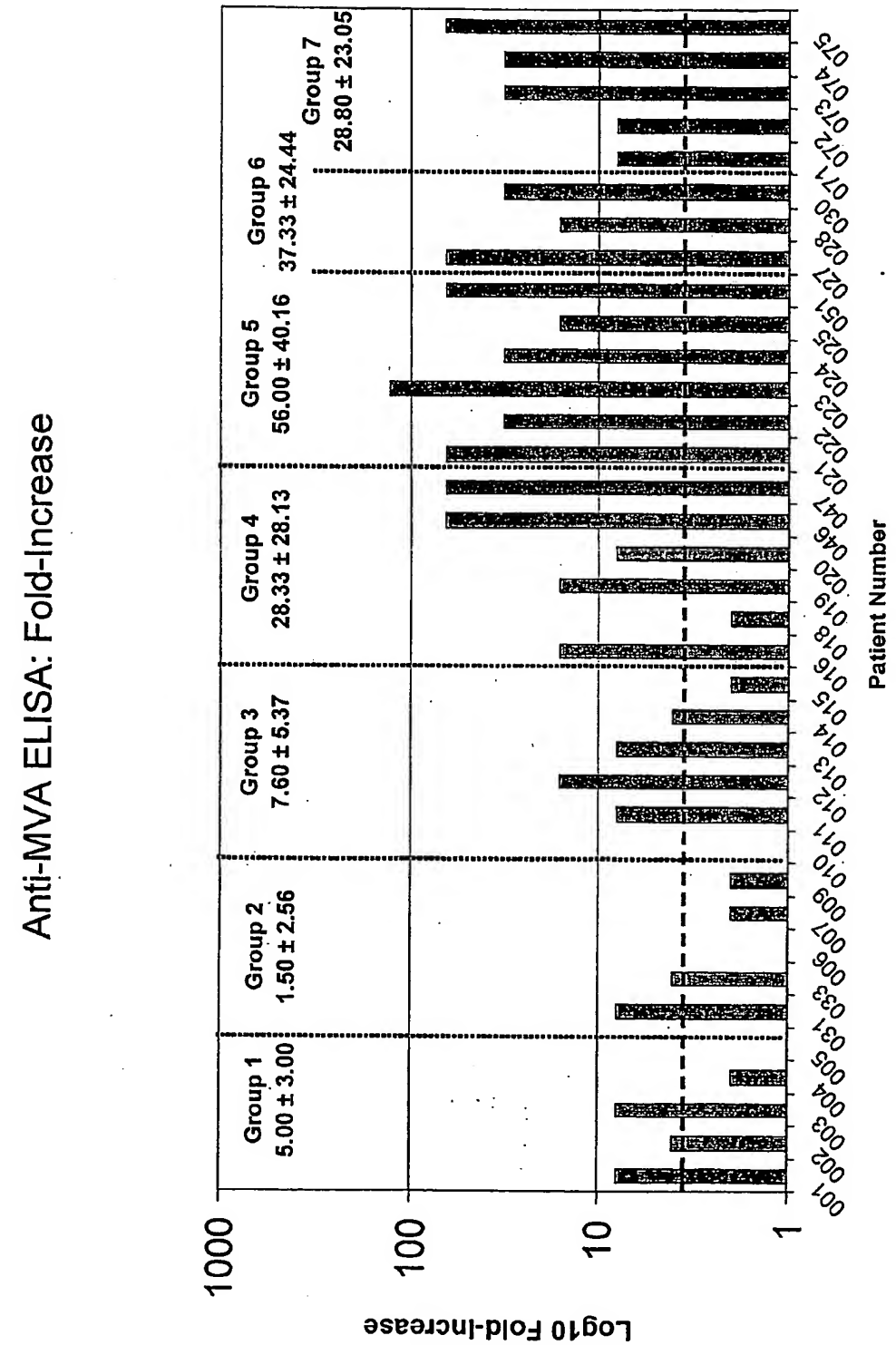

Anti-MVA ELISA: Fold-Increase
Group 1
5.00 ± 3.00
Group 2
1.50 ± 2.56
Group 3
7.60 ± 5.37
Group 4
28.33 ± 28.13
Group 5
56.00 ± 40.16
Group 6
37.33 ± 24.44
Group 7
28.80 ± 23.05
1000
100
10
1
Log10 Fold-Increase
001 002 003 004 005 031 033 006 007 009 010 011 012 013 014 015 016 018 019 020 046 047 021 022 023 024 025 051 027 028 030 071 072 073 074 075
Patient Number

Figure 29

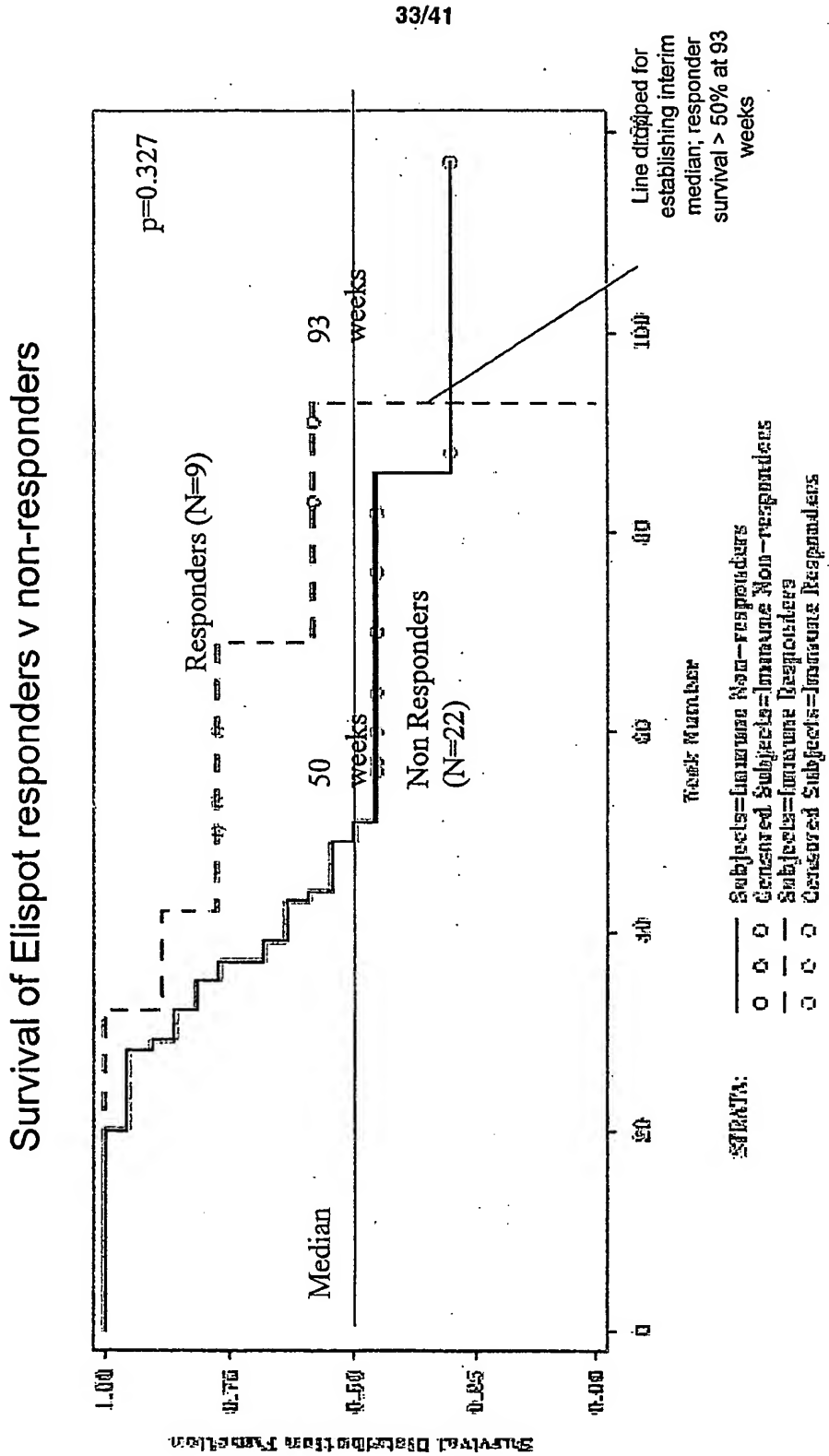

Survival of Elispot responders v non-responders
p=0.327
Responders (N=9)
Non Responders (N=22)
Median
50
weeks
93
weeks
Line dropped for establishing interim median; responder survival > 50% at 93 weeks
Survival Distribution Function
1.00
0.75
0.50
0.25
0.00
0
20
40
60
80
100
Week Number
DATA:
Subjects=Immune Non-responders
Censored Subjects=Immune Non-responders
Subjects=Immune Responders
Censored Subjects=Immune Responders

Figure 30

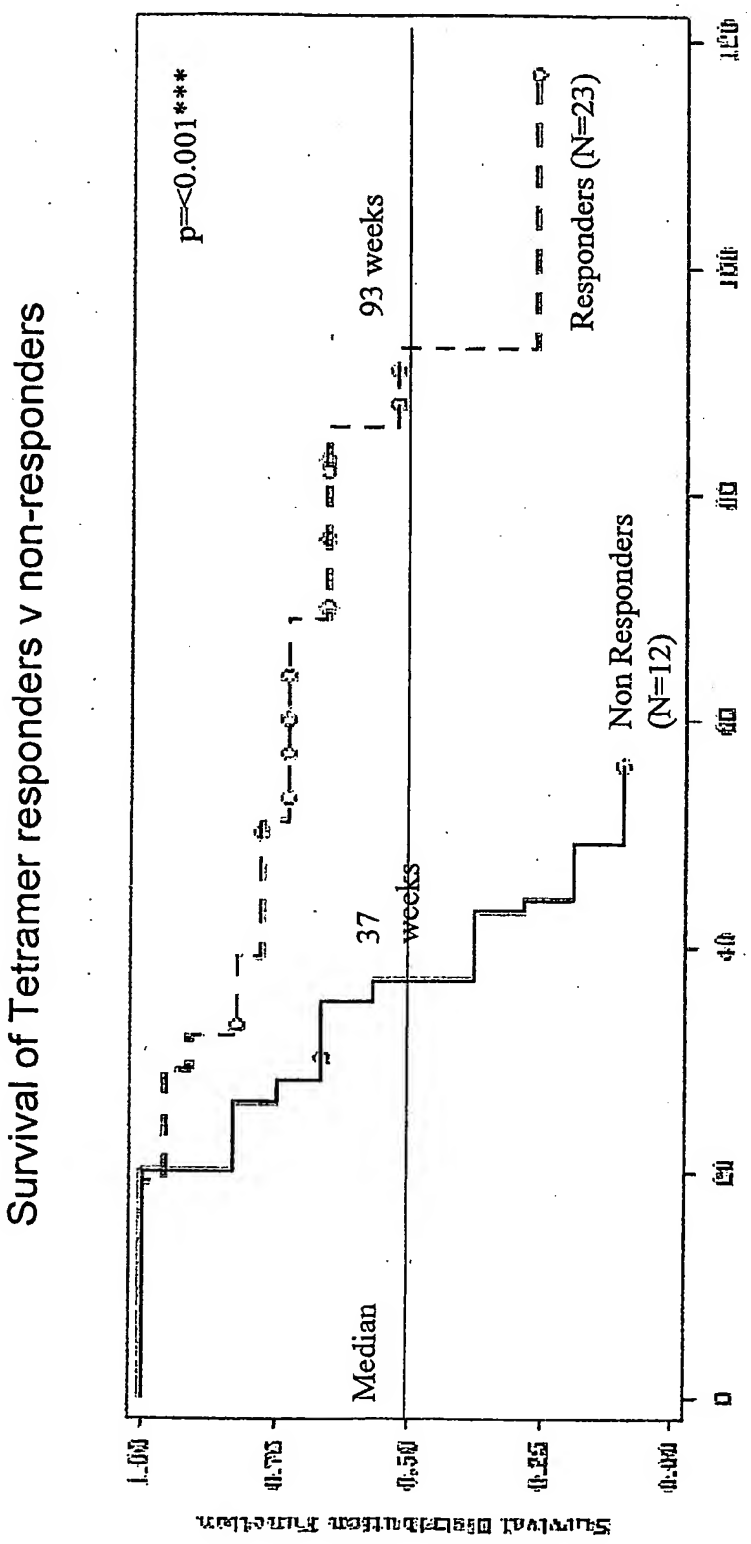

Survival of Tetramer responders v non-responders
p=<0.001***
Median
37
weeks
93 weeks
Responders (N=23)
Non Responders
(N=12)
Survival Distribution Function
1.00
0.75
0.50
0.25
0.00
0
20
40
60
100
120
Week Number
STRATA:
Subjects=Tetramer Immune Non-responders
Censored Subjects=Tetramer Immune Non-re
Subjects=Tetramer Immune Responders
Censored Subjects=Tetramer Immune Respon

Figure 31

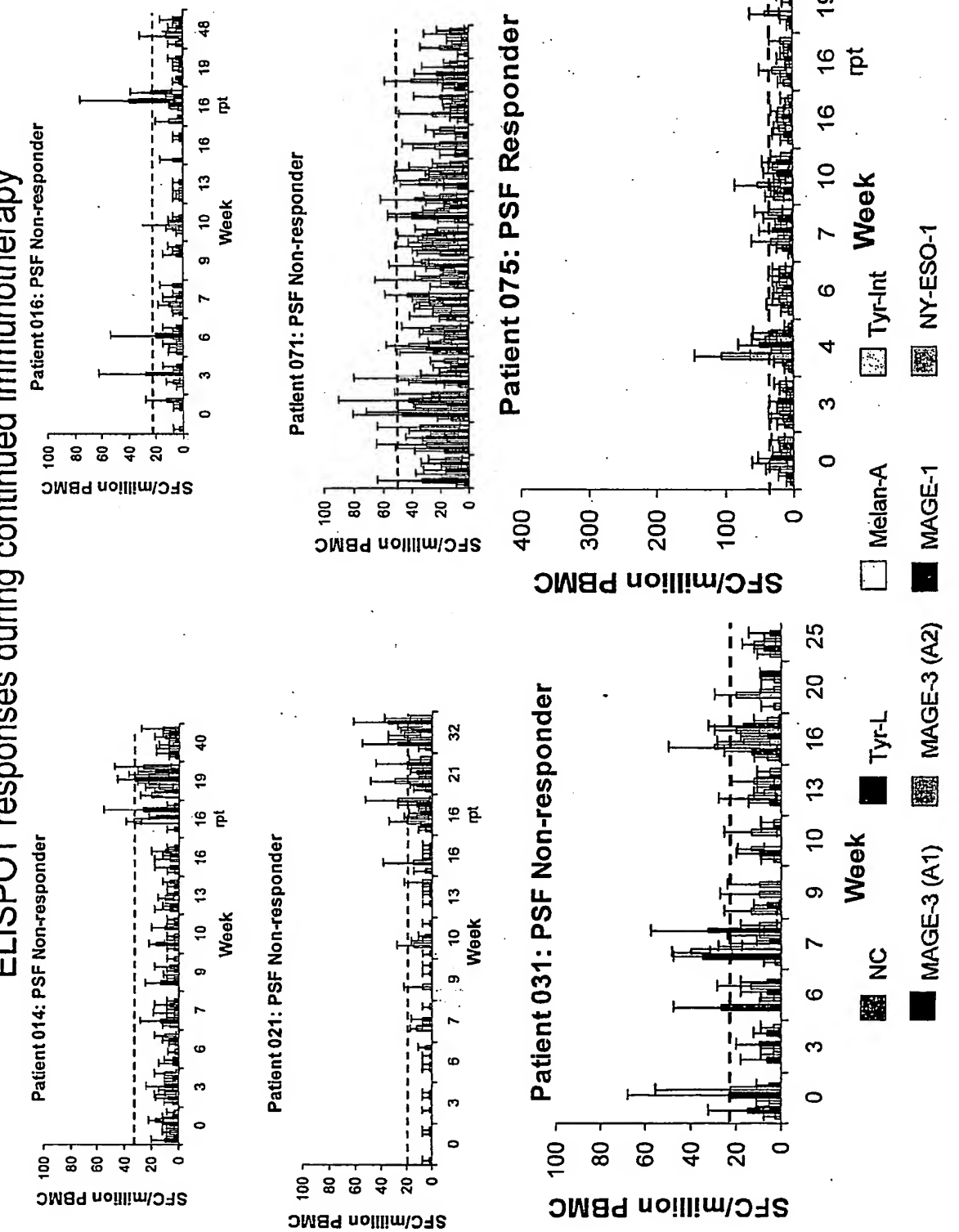

ELISPOT responses during continued immunotherapy
Patient 014: PSF Non-responder
Patient 016: PSF Non-responder
Patient 021: PSF Non-responder
Patient 031: PSF Non-responder
Patient 071: PSF Non-responder
Patient 075: PSF Responder
SFC/million PBMC
Week
rpt
NC
Tyr-L
Melan-A
Tyr-Int
MAGE-3 (A1)
MAGE-3 (A2)
MAGE-1
NY-ESO-1

Figure 32

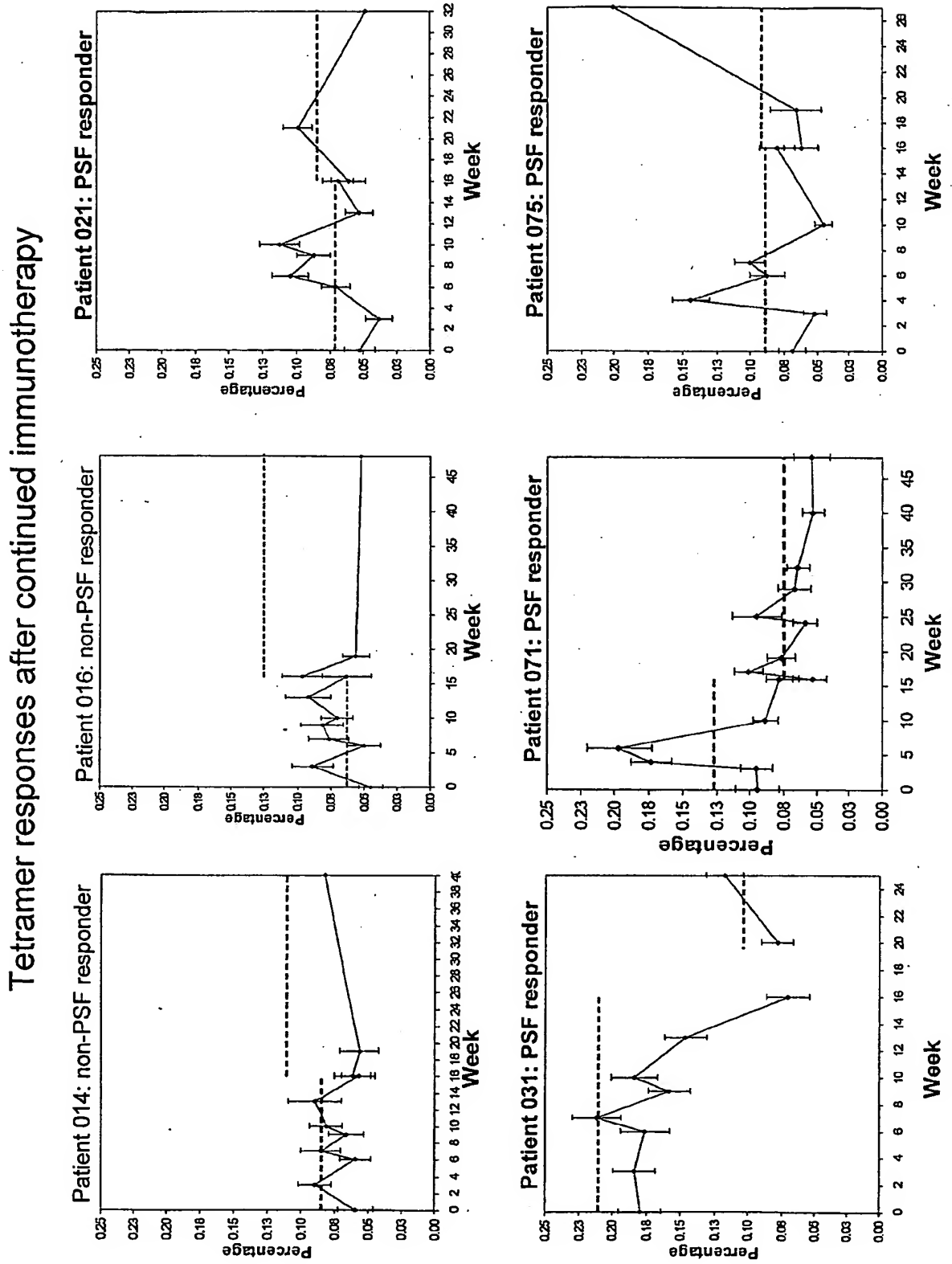

Tetramer responses after continued immunotherapy
Patient 014: non-PSF responder
Patient 016: non-PSF responder
Patient 021: PSF responder
Patient 031: PSF responder
Patient 071: PSF responder
Patient 075: PSF responder
Percentage
Week

Figure 33

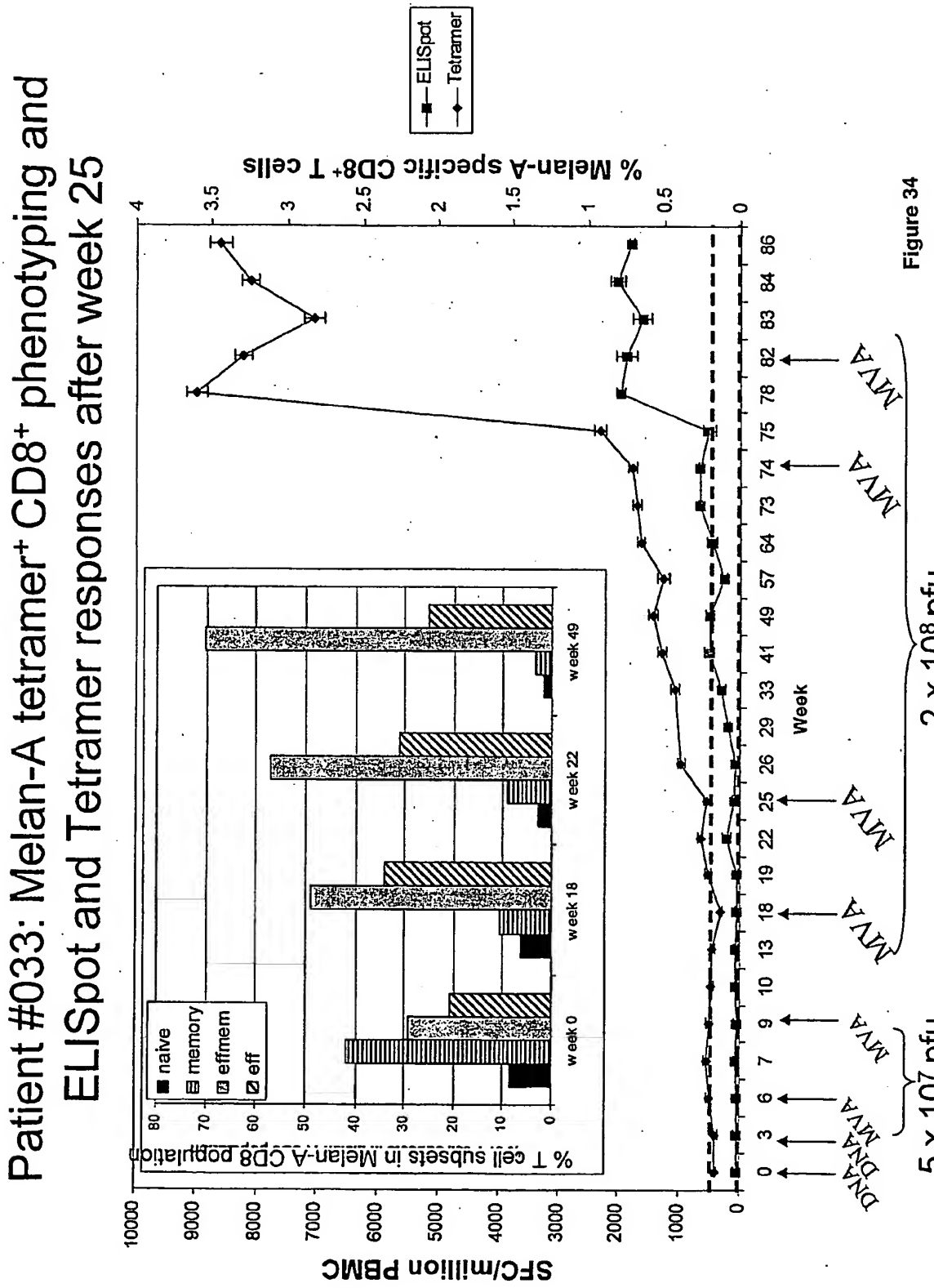
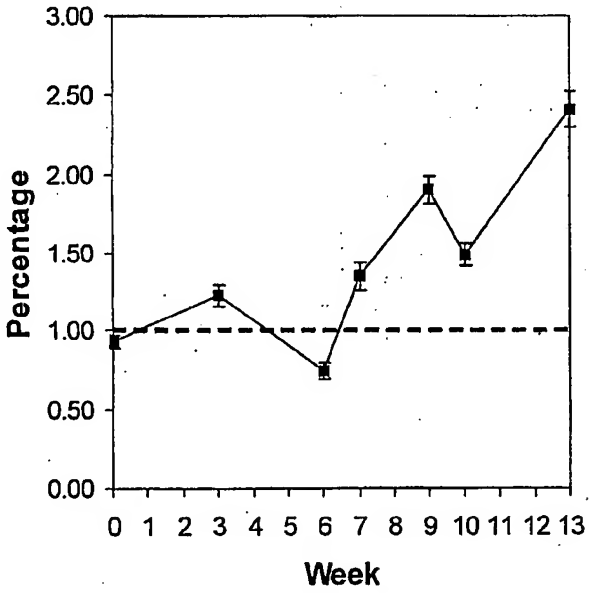

Patient #033: Melan-A tetramer+ CD8+ phenotyping and ELISpot and Tetramer responses after week 25
SFC/million PBMC
% Melan-A specific CD8+ T cells
ELISpot
Tetramer
% T cell subsets in Melan-A CD8 population
naive
memory
effmem
eff
week 0
week 18
week 22
week 49
Week
DNA
MVA
5 x 10^7 pfu
2 x 10^8 pfu

Figure 34

Patient #047: Melan-A tetramer^{+} CD8^{+} phenotyping

A



B

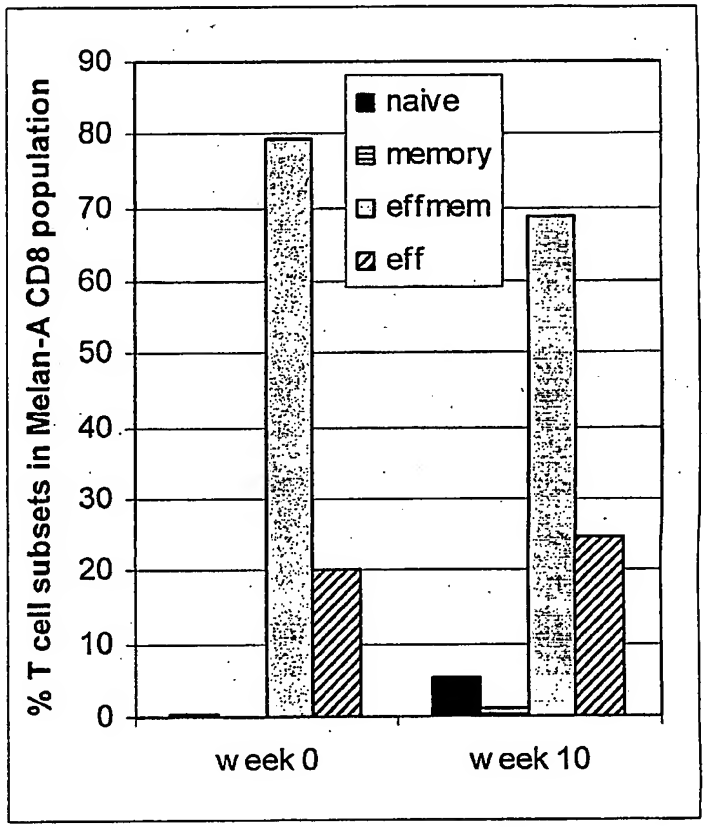


Figure 35

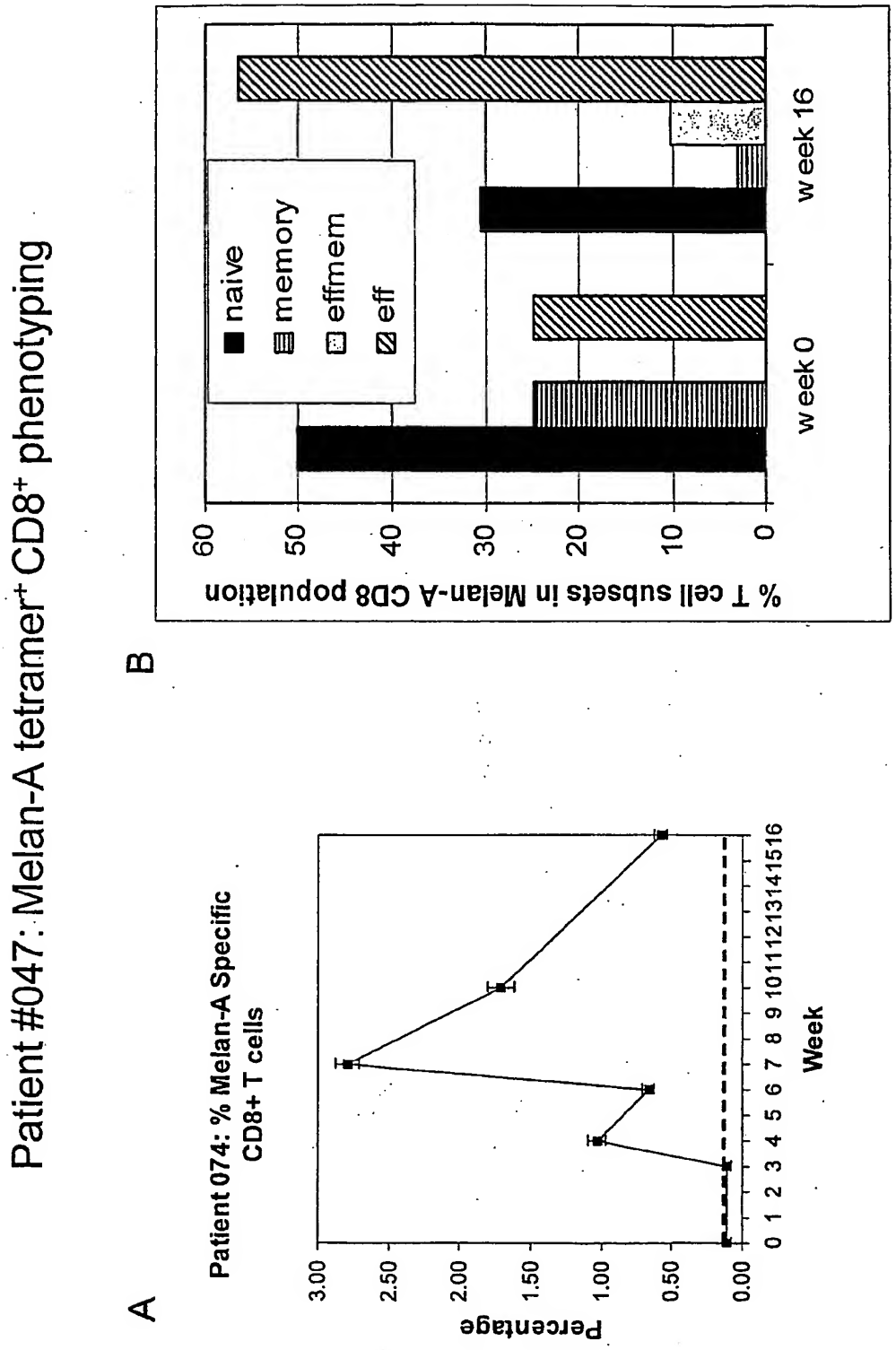

Patient #047: Melan-A tetramer+ CD8+ phenotyping
A
Patient 074: % Melan-A Specific CD8+ T cells
Percentage
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Week
B
% T cell subsets in Melan-A CD8 population
60
50
40
30
20
10
0
naive
memory
effmem
eff
week 0
week 16

Figure 36

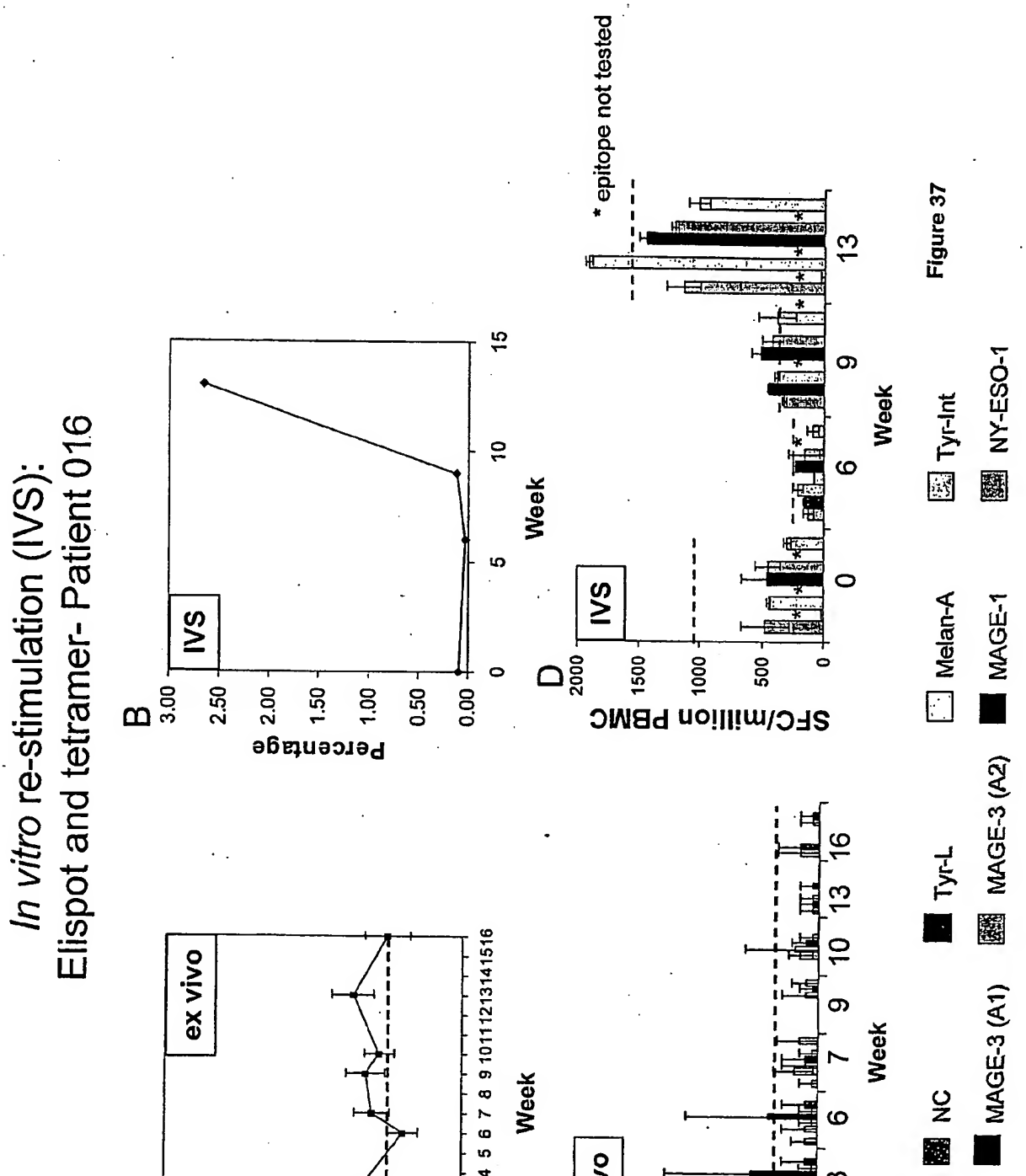

In vitro re-stimulation (IVS):
Elispot and tetramer- Patient 016
A
ex vivo
Percentage
Week
B
IVS
Percentage
Week
C
Ex vivo
SFC/million PBMC
Week
D
IVS
* epitope not tested
SFC/million PBMC
Week
NC
Tyr-L
Melan-A
Tyr-Int
MAGE-3 (A1)
MAGE-3 (A2)
MAGE-1
NY-ESO-1

Figure 37

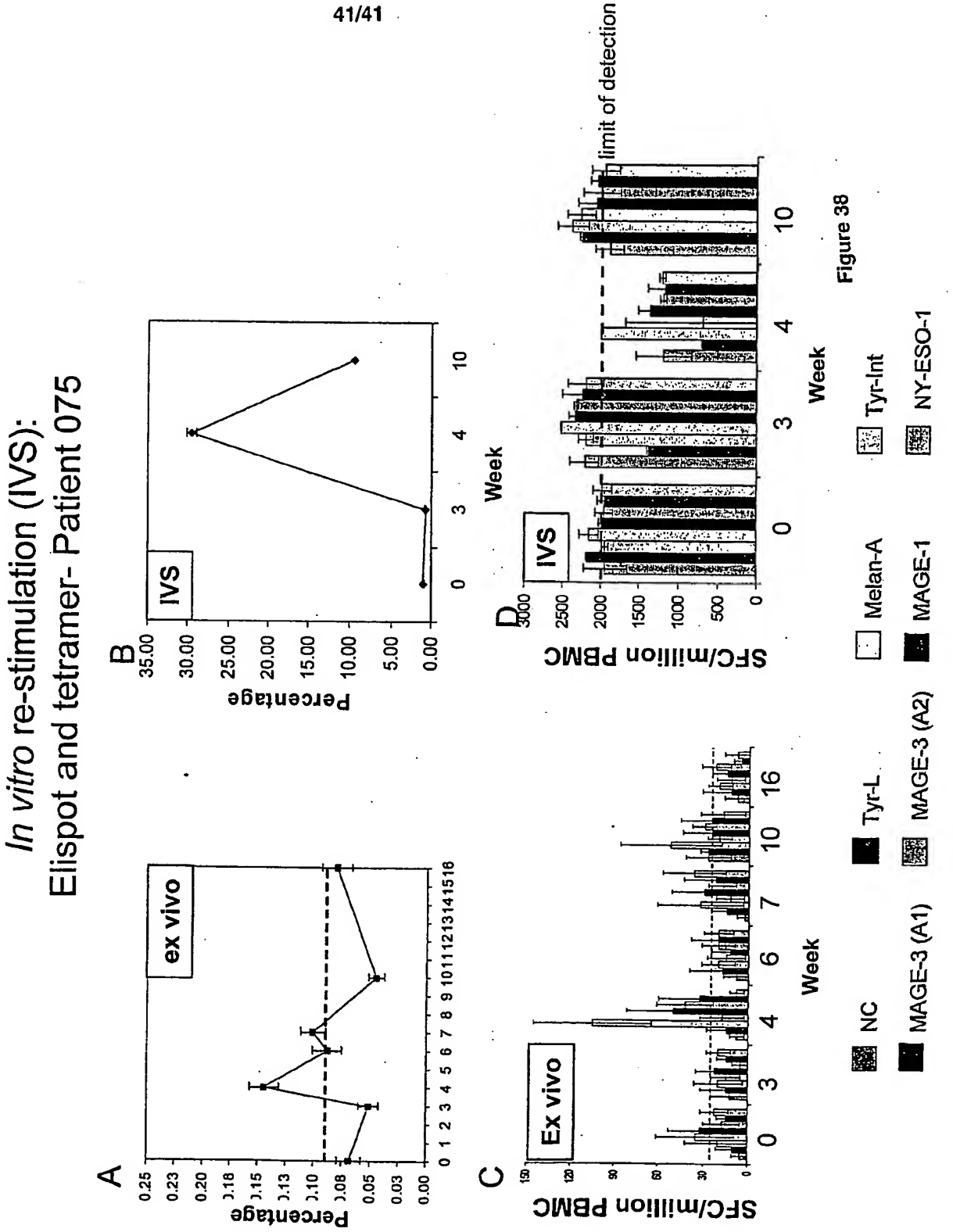

In vitro re-stimulation (IVS):
Elispot and tetramer- Patient 075
A
ex vivo
Percentage
B
IVS
Percentage
Week
C
Ex vivo
SFC/million PBMC
Week
D
IVS
SFC/million PBMC
limit of detection
Week
NC
Tyr-L
MAGE-3 (A1)
MAGE-3 (A2)
Melan-A
Tyr-Int
MAGE-1
NY-ESO-1

Figure 38

VIII-5-1	**Declaration: Non-prejudicial disclosure or exceptions to lack of novelty** Declaration as to non-prejudicial disclosures or exceptions to lack of novelty (Rules 4.17(v) and 51bis.1(a)(v)): Name (LAST, First)	in relation to this international application OXXON THERAPEUTICS LTD declares that the subject matter claimed in this international application was disclosed as follows:
VIII-5-1(i)	Kind of disclosure:	other Oral Presentation With Slides
VIII-5-1(ii)	Date of disclosure:	06 April 2006 (06.04.2006)
VIII-5-1(iii)	Title of disclosure:	"Mad Dogs and Englishmen - An Update on Melanoma and its Treatment"
VIII-5-1(iv)	Place of disclosure:	